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# The Effect of Atropine on the Acidity of a Denervated Gastric Pouch.

By

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Atropine has been a widely used drug to depress the gastric secretion. Its effect is very marked on secretion that has been induced by vagal stimulation (cephalic phase), or by food, but even after stimulation with histamine an inhibition can be observed (cf. POLLAND 1930, GRAY 1937, CORSI and SANGIORGI 1954). This inhibition has been established for both the volume output and the total acid output, but very few observations have been reported on the effect of atropine on the *acidity* of the gastric juice. In cases where this problem has been treated it has sometimes been found that the acidity does not decrease after atropine but very often increases. KEETON, LUCKHARDT and KOCH (1920) in a total gastric pouch and a Pavlov pouch noticed an acidity reduction, that was much less than the inhibition of the volume secretion, and also they found a time lag between the decrease in volume and acidity. In man POLLAND (1930) after a subcutaneous injection of histamine found a much more marked inhibition of volume than acid output due to an increase in acidity. GRAY (1937) occasionally made similar observations. NOTKIN, FORSE and WEBSTER (1953) on dogs with intact vagus innervation found a decrease in volume after bellafoline but an unchanged pH. Their results do not confirm an earlier finding of BENJAMINE, ROSIERE and GROSSMAN (1950) that atropine markedly increased the pH which occurred after a considerable time lag.

It is known that some of the non acid components of the gastric juice, *i. e.* pepsin and mucus are stimulated by the vagus directly (LINDE 1950, GLASS and BOYD 1950).

The mode of atropine action is thought to be an inhibition of the effect of acetylcholine on the effector organs. Consequently it might be concluded that the tendency of atropine to increase the gastric acidity, should be mainly due to an inhibition of the non acid components. This would fit in with the theories of PAVLOV (1898) and others that the main factor for the acidity regulation is dilution and neutralisation with a non-partial secretion (cf. HOLLANDER 1952, BABKIN 1954).

In the case of a denervated gastric pouch an administration of atropine should not be expected to have so great an influence on the acidity as the secretion of non acid components is always very low. Nevertheless a dissociation has been demonstrated between the volume output and the acidity after atropine also in denervated gastric pouches (HOLLANDER 1952). From his experiments HOLLANDER seemed to conclude that a close relationship between volume output and acidity does not exist, although such a relationship has frequently been stressed (cf. BUCHER, IVY and GRAY 1941, GRAY, BUCHER and HARMAN 1941, TEORELL 1947, ÖBRINK 1948, HEINZ and ÖBRINK 1954). According to TEORELL (1933, 1947) this relationship should be due to a diffusion like process that regulates the acidity in relation to the volume output and could be described as

$$H^+ = \frac{C_o}{\frac{k}{v} + 1} \qquad (1)$$

where $H^+$ is the actual hydrogen ion concentration (mN), $C_o$ the primary acidity (mN), $k$ a permeability coefficient (ml/min) and $v$ the secretion rate (ml/min). This formula was found by ÖBRINK (1948) to describe rather well the existing relationship in Heidenhain pouch dogs. In this latter paper, however, it was pointed out that after an inhibition of the volume output the acidity does not immediately decrease. A time lag always exists because some fluid adheres to the mucous surface in the crypts and in the cannula (the 'lake-hypothesis'). Thus it seems probable that a definite relationship between volume output and acidity is experimentally well established, and that the dissociation of the two parameters after atropine found by HOLLANDER (1952) and others

is due to either *a*) the time lag between volume inhibition and acidity decrease which exists normally or *b*) a change in the quantitative properties of the acidity regulation. In the case of a denervated pouch this means a change in the permeability coefficient for the hydrogen ions.

It is the purpose of this paper to try to test which of these explanations is the most probable.

## Methods.

The experiments were carried out on Heidenhain pouch dogs and and on cats under Nembutal ® (Abbott) anaesthesia. The animals were starved for at least twelve hours.

In the *dog experiments* secretion was induced by a continuous intravenous injection of histamine that gave a secretion of about 50 % of the maximal secretion rate, and when the volume secretion had reached a steady state, atropine was injected also by the continuous intravenous route.

In the *cat experiments* ligatures were passed round the pylorus and the cardia. Thus both vagi were functionally sectioned, the stomach was at complete rest when a known amount of 170 mN HCl was instilled into it (about 7 ml). Every fifteen minutes this volume was taken out and measured and a 0.1 ml aliquot was taken for analysis. The rest was poured back into the stomach and left for another fifteen minutes. This procedure was repeated throughout the experiment. (For details in the technique see TEORELL 19.33)

After about 90 minutes the experiment was interrupted. Atropine was then given to the cat subcutaneously, and after about 20 minutes a fresh volume of 170 mN HCl was instilled and the same procedure again repeated.

## Results.

### The Cat Experiments.

*Figure 1* shows a typical experiment with instillation of HCl. The acidity declined in an exponential way. According to the diffusion theory this decrease would be described by:

$$H = H_o \; e^{-kt/p} \qquad (2)$$

where $H$ and $H_o$ are the acidity values (mN) at time $t$ and zero, $k$ the permeability coefficient (ml/min) and $p$ the volume instilled (ml). Rearrangement of equation (2) gives:

$$\log \frac{H}{H_o} = - \frac{0.4343 \; k \; t}{p} \qquad (3)$$

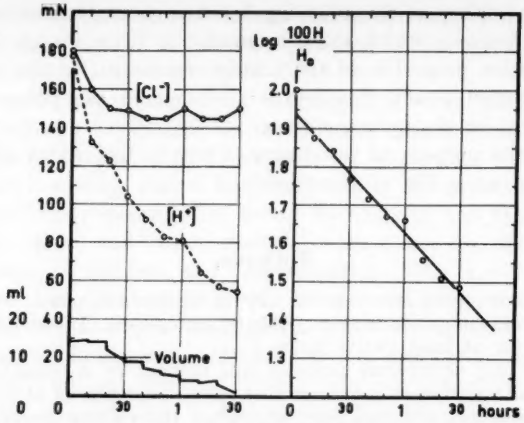


Fig. 1. Instillation of HCl in a cat's stomach. The acidity curves in the left (linear scale) and right (logarithmic scale) figure show the exponential fall.

As $p$ and $k$ are constants equation (3) describes a straight line representing the relationship between $\log \frac{H^o}{H_o}$ and $t$. The slope of this line is $-\frac{0.4343\ k}{p}$ and if the volume $p$ is known, $k$ can be calculated from the observed slope.

*Figure 2* shows a typical experiment with one period before and one after atropine. The curves demonstrate the relation between $\log \frac{H_o}{H_o}$ and $t$ according to eq. (3). The resulting $k$-values are given in *table I* where the permeability coefficient in the control instillation $= k_1$ and that after atropine $= k_2$. In every experiment $k_1$ was found to be greater than $k_2$ indicating a lower rate for the disappearance of hydrogen ions from the gastric contents after atropine. The decreases from $k_1$ to $k_2$ expressed as percentages of $k_1$ are seen in the right hand column in table I. They yield a mean decrease of $14.4 \pm 2.29$ % (mean $\pm$ standard error of the mean). This average decrease is thus significantly greater than zero ($P < 0.001$).

### The Dog Experiments.

In these experiments in which secretion was induced by histamine it was not possible to use the experiment on the same day

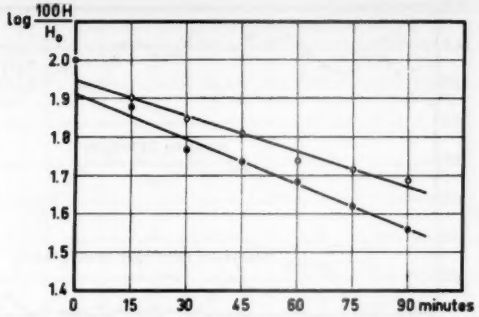


Fig. 2. Instillation of HCl in a cat's stomach. There is a slight difference in slope before (•) and after (○) atropine.

**Table I.**

*The permeability coefficients for $H^+$ ions in the cat's stomach before ($k_1$) and after ($k_2$) atropine.*

| Experiment nr. | Permeability coefficient | | $\frac{(k_1 - k_2) \times 100}{k_1} = d$ |
|---|---|---|---|
| | control $k_1$ | after atropine $k_2$ | |
| 1 | 0.0498 | 0.0468 | 6.02 |
| 2 | 0.0660 | 0.0487 | 26.21 |
| 3 | 0.0747 | 0.0656 | 12.18 |
| 4 | 0.0787 | 0.0656 | 16.65 |
| 5 | 0.0819 | 0.0726 | 11.36 |
| 6 | 0.0651 | 0.0485 | 25.50 |
| 7 | 0.0632 | 0.0548 | 13.29 |
| 8 | 0.0608 | 0.0564 | 7.24 |
| 9 | 0.0466 | 0.0402 | 13.73 |
| 10 | 0.0720 | 0.0634 | 11.94 |

$d = 14.4 \pm 2.29$.

as control for the next experiment with atropine as it was in the instillation experiments on cats. This was because in secretion experiments the permeability coefficient must be calculated from:

$$H = \frac{C_o}{\frac{k}{v} + 1}$$ (eq. (1)) which is obtainable only from several steady

state experiments. A decrease in volume secretion and generally also in the acidity after a certain time lag was found (fig. 3). (In some experiments, however, an increase in acidity was re-

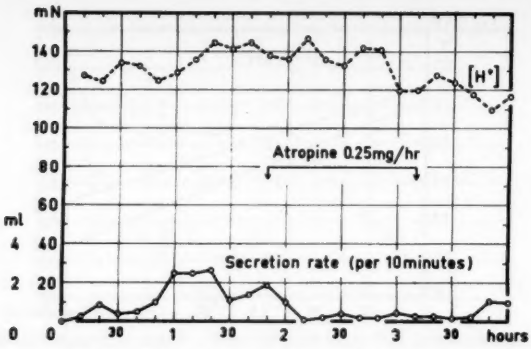


Fig. 3. Secretion in a pouch dog after continuous histamine stimulation. Atropine caused an immediate decrease in volume and a later decrease in acidity.

corded (fig. 4). After the lag time the acidity attained a new steady state value. A comparison of the relation between the volume and the acidity after atropine with the normal relationship for the same dog did not allow a definite conclusion that there were differences in the value of $k$.

## Discussion.

It could be confirmed that in secreting gastric pouches (histamine stimulation) atropine causes a decrease in volume secretion rate and generally also in the acidity. The decrease in volume, however, comes much sooner than that of the acidity. This time lag is not an effect of the atropine but depends on the 'lake' volumes in the pouch (cf. ÖBRINK 1948, p. 63). As can be seen in fig. 3 the acidity normally decreases if sufficient time is allowed. Thus great care must be taken in drawing any conclusions about the relationship between volume and acidity immediately after the administration of atropine, because it takes time for a steady state to develop. If this factor was considered it was observed that the normal relationship between the volume and acidity was only occasionally disturbed as in the experiments of HOLLANDER (1952). Such experiments, however, seem to indicate a decreased $k$-value after atropine, thus confirming the findings of HOLLANDER.

Instillation experiments on cats, where the vagi were ligated, and where no secretion existed, showed a diminished rate of dis-

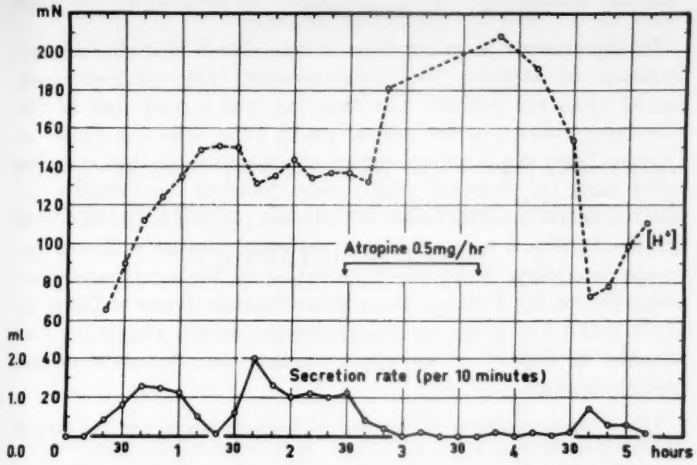


Fig. 4. Secretion in a pouch dog after continuous histamine stimulation. In this experiment an increase in acidity was observed despite the decrease in secretion rate.

appearance of hydrogen ions from the instilled solution, which probably was due to a decreased permeability for the hydrogen ions through the gastric mucosa and was not likely to be due to a decreased production of gastric mucus and pepsin. A decreased permeability through the gastric mucosa could be explained if the atropine was thought to result in a vasoconstriction in the gastric mucosa.

Wolf and Wolff (1943) Vand ane and Thompson (1952) have shown the influence of the blood flow on the gastric secretion. Nordgren and Öbrink (1956) suggested that the degree of vascularisation of the mucosa influenced upon the maximal secretion rate of the fundus cells. The present experiments seem to indicate a similar action of the vascularisation on the acidity regulation. This implies, that any substance that interferes in either direction with the blood flow in the gastric mucosa would alter the normal relationship between the volume output and acidity. Experiments are in progress where substances known to constrict or dilate the vascular bed are used, and where the actual vascularisation is studied.

## Summary.

In experiments in pouch dogs it was shown that after administration of atropine the volume secretion rate declines much sooner than the acidity. The time lag was mainly due to the remaining volume in the gastric pouch (The *'lake hypothesis'* of ÖBRINK 1948). Earlier observations of other authors that atropine could cause an increased acidity were, however, confirmed especially in cats where HCl was instilled and a permeability coefficient for the hydrogen ions calculated. Atropine caused a slower decrease in acidity, which could be explained by a decreased permeability for the hydrogen ions. (The diffusion theory of TEORELL 1933, 1947.) The action of atropine on the acidity regulation was thought to depend on its effect on the vascularisation of the gastric mucosa.

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# The Available Glycogen in Man and the Connection between Rate of Oxygen Intake and Carbohydrate Usage.

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Knowledge of the amount of carbohydrate which can be stored in the human body and made available under various conditions of reduced food intake is of considerable practical importance in deciding the necessary rations required by military forces and also for athletes engaged in endurance performances in sport.

The normal fat storage content of the body itself would provide a sufficient energy depot for long-lasting survival even in starvation. Unfortunately, however, a certain fraction of the energy output has to be covered by carbohydrate oxidation if ketosis and hypoglycemia is to be prevented, and during heavy exercise this fraction is maintained at a high level, even though the glycogen stores are considerably depleted with resultant hypoglycemic symptoms (CHRISTENSEN and HANSEN 1939).

As a direct determination of the glycogen content in man is impossible, indirect techniques are required. One method is to determine the carbohydrate content in other mammals through analyzes of the different organs, liver, muscles etc. and to estimate the corresponding values for man. Based on this type of calculations WRIGHT (1945) gives the following figures: "About 500 g of glycogen are, on an average, stored in the body, about 100 g in the liver, and practically all the rest in the skeletal muscles." according to BEST and TAYLOR (1955) the muscles contain between 0.2—1.8 % glycogen and the liver 0.2—10 %. Assuming that these figures are valid for man, we have calculated the maximal values for those who served as subjects in this investigation (average weight 72 kg) to about 700 g of glycogen.

**Table 1.**

*Age, height, weight and aerobic capacity of the subjects.*

| Subject | Age years | Body height cm | Body weight kg | Max. $O_2$ intake l/min[1] (STPD) | Max. $O_2$ intake per kg ml/min |
|---|---|---|---|---|---|
| M. C. ..... | 23 | 186 | 76.7 | 4.76 | 62.1 |
| R. H. ..... | 25 | 177 | 73.5 | 4.78 | 65.0 |
| A. K. ..... | 28 | 171 | 67.2 | 4.25 | 63.2 |
| S. M. ..... | 35 | 174 | 71.2 | 4.88 | 68.5 |

[1] attained during ski running.

A second method, which we ourselves have used here, is based on an accurate determination of the respiratory metabolism. In this way the minimum values for the available glycogen reserves are obtained. By this method BENEDICT and CATHCART (1913) computed from their work experiment on the bicycle ergometer a value of 368 g of metabolized glycogen. CHRISTENSEN and HANSEN (1939) also chose this method. With a work intensity which gave an oxygen intake of 2.9 l/min, they calculated the available glycogen depot, after an extreme increase in the carbohydrate diet, to be at least 400 g.

In our investigation with long distance ski running an appreciably higher work intensity was chosen; the oxygen intake was 3.6—4.1 l/min. The local sensation of exertion in ski running is slight, even when the metabolism is high, since the total area of muscle cross-sections over which the work load (*i. e.* oxygen supply) is distributed, is very large. For this reason also, localized muscle fatigue is of lesser degree than in many other forms of athletics and therefore allows a prolonged work period, which is of great importance for a study of this type.

## Method.

The subjects, all well-trained skiers, consisted of three male students at a college of physical education: M. C., 23 years old, R. H., 25 and A. K., 28; a fourth subject was S. M., 35 years old and a competition skier of the Swedish élite class (winner in the Vasa-race, 85 km, 1952). Data on the subjects including age, height, weight and maximal oxygen intake are shown in table 1.

The day before the experiment, up to 10 o'clock p. m., the subjects ate as much as they desired, particularly carbohydrates. They were

Fig. 1. Skiing with Douglas bag.

not allowed to do any heavy muscular work, in order that the body glycogen depot would be kept untouched. They slept overnight in the laboratory and basal values for pulse rate, oxygen intake and respiratory quotient as well as the blood sugar concentration were determined before rising in the morning. Two determinations of the oxygen intake were made with the Douglas bag method, and the mean value of these two figures was used.

When the ski running began, the subjects had been fasting at least for the previous 12 hours. The experiments were performed on a 750 m circular track set up on level ground. The time for every lap around the track was measured and told to the subjects so that they would try to maintain a constant speed during the entire experiment. The subjects ran with the Douglas bag attached to a special device carried on the back, which did not hinder skiing (fig. 1). When oxygen intake was not being determined, a rucksack of corresponding weight, 5.4 kg, was carried in place of the Douglas bag. Two oxygen intake determinations were made for every 10 laps (about 30 min) in the following way (see also fig. 2): after the subject had run 4,500 m (18—19 min) with the rucksack, he stopped for 1 to 1 $^1/_2$ min and the Douglas bag was fastened to his back. During the following 4 $^1/_2$ to 5 min (about 1,200 m) he breathed through the valve. Thereafter during continuous skiing the three-way stop cock on the Douglas bag was turned on and the expired air was collected during 1 to 1 $^1/_2$ min (about 300 m). After changing the bag, which took $^1/_2$ min, this procedure was repeated during continuous running for 1,500 m. Immediately when the subject stopped, in connection with each changing of the bag, the time for 10

Table

*Daily routine for subject*

| Date | Hour | Pulse rate | $O_2$ intake l/min STPD | RQ | Blood sugar conc. mg % | Urine | |
|---|---|---|---|---|---|---|---|
| | | | | | | amount ml | $N_2$ conc. mg/ml |
| 2/2 | 9—21 | | | | | 590 | 15.15 |
| 2—3/2 | 21—9 | | | | | 575 | 17.78 |
| 3/2 | 7 | 45 | 0.28 | 0.84 | 96 | | |
| » | $9^{35}$ — $12^{15}$ | 158—176 | 3.75—4.11 | 0.87—0.91 | | | |
| » | $12^{15}$ | | | | 89 | | |
| » | $13^{00}$ — $13^{30}$ | 60 | 0.34 | 0.75 | 83 | | |
| » | $9^{00}$ — $13^{45}$ | | | | | 140 | 16.82 |
| » | $15^{00}$ — $15^{30}$ | 53 | 0.31 | 0.75 | | | |
| » | $16^{30}$ | 110 | 1.60 | 0.73 | 76 | | |
| » | $18^{30}$ — $19^{00}$ | 46 | 0.29 | 0.71 | | | |
| » | $12^{15}$ — 19 | | | | | | |
| » | $13^{45}$ — 21 | | | | | 180 | 16.75 |
| » | 22 | | | | | | |
| 3—4/2 | 21—9 | | | | | 250 | 26.41 |
| 4/2 | 7 | 46 | 0.29 | 0.74 | | | |
| » | $7^{30}$ | 105 | 1.60 | 0.75 | 80 | | |
| » | 9 | | | | | | |
| » | 9—21 | | | | | 350 | 26.37 |
| 4—5/2 | 21—9 | | | | | 435 | 25.79 |
| 5/2 | 9 | | | | 114 | | |
| » | 9—21 | | | | | 465 | 15.70 |
| 6/2 | 9 | 96 | 1.49 | 0.86 | | | |

2

*R. H. 2/2—6/2 1954.*

| analyses | | | Remarks |
|---|---|---|---|
| $N_2$ amount g | Acetone | Protein | |
| 8.94 | | | Day urine, "normal day". |
| 10.22 | | | Night urine, "normal day". |
| | | | Determination of basal metabolism & blood sugar conc. |
| | | | Ski running 35.25 km (47 × 750 m) with a 5.4 kg load (during fasting). |
| | | | Blood lactic acid 19 mg %. |
| | | | Determination of pulse, $O_2$ intake, RQ & blood sugar conc. at rest. |
| 2.36 | neg. | pos. | Day urine I (during ski running). |
| | | | Determination of pulse, $O_2$ intake & RQ at rest. |
| | | | Cycling 600 kpm/min for 8 minutes. |
| | | | Determination of pulse, $O_2$ intake & RQ at rest. |
| | | | Water intake $^1/_2$ l. No food. |
| 3.02 | pos. | neg. | Day urine II. |
| | | | To bed. |
| 6.60 | pos. | neg. | Night urine. |
| | | | Determination of basal metabolism. |
| | | | Cycling 600 kpm/min for 8 minutes. |
| | | | First meal on normal food. |
| 9.23 | | | Day urine. |
| 11.22 | | | Night urine. |
| | | | Determination of blood sugar conc. |
| 7.30 | | | Day urine. |
| | | | Cycling 600 kpm/min for 8 minutes. |

pulse beats was measured. The experiment was stopped when the subject became so exhausted that he was not able to keep up a reasonably constant speed. Blood samples were taken immediately after conclusion of the experiment in order to determine the concentration of blood lactic acid (according to Edward's method) and blood sugar (according to Creselius-Seifert).

In order to follow the process of recovery after exercise, 2 to 3 determinations of the pulse rate, the respiratory metabolism and blood sugar concentration were determined during the afternoon at intervals of two to four hours. The subjects were still fasting but were allowed to drink water *ad libitum*. A few hours after ski running the subjects performed a test work of 600 kpm/min for 8 min on the bicycle ergometer.

The determinations of basal metabolism and blood sugar concentration, followed by the ergometer test, were repeated in the morning the day after the ski experiment, after which time the subjects were allowed to return to their normal eating habits.

The total nitrogen content of the urine samples were determined according to Kjeldahl's macro-method. The presence of acetone in the urine was examined by Legal's test, and proteinuria determined by Sörensen's reagent.

Each experiment was performed similarly to that shown in table 2 for R. H., with a pre-arranged daily routine. Weather and snow conditions were fairly similar throughout the series with a temperature of 3°—6° C below freezing point and little wind. In general, gliding conditions got worse (because of lack of ski-wax *etc.*) during the second half of the experiment.

The aerobic capacity, *i. e.* the maximal oxygen intake capacity, of the different subjects was determined during ski running. After a period of active warming up the subject ran at maximal speed, during which time he breathed through the valve for 5 min and the expired air was collected during the following min. The high blood lactic acid concentrations ($\geqq$ 100 mg %) harmonize with our belief that the recorded oxygen intakes, given in table 1, are sensibly maximal.

## Results.

### A. Ski Running.

*Amount of work, pulse rate, pulmonary ventilation, oxygen intake and blood lactic acid.* Subject S. M. ran 42 km with a mean speed of nearly 16 km/hour; R. H. and M. C., 35 km each, and A. K. 28 km, all with a speed of about 14 km/hour. The pulse rate for each subject during the whole running time was in the range of 160—190 beats/min, and the pulmonary ventilation (BTPS) around 90 l/min. During the 2 $^1/_2$ hours' long experiment R. H. and M. C. consumed on an average 3.9 l $O_2$/min, which implies that

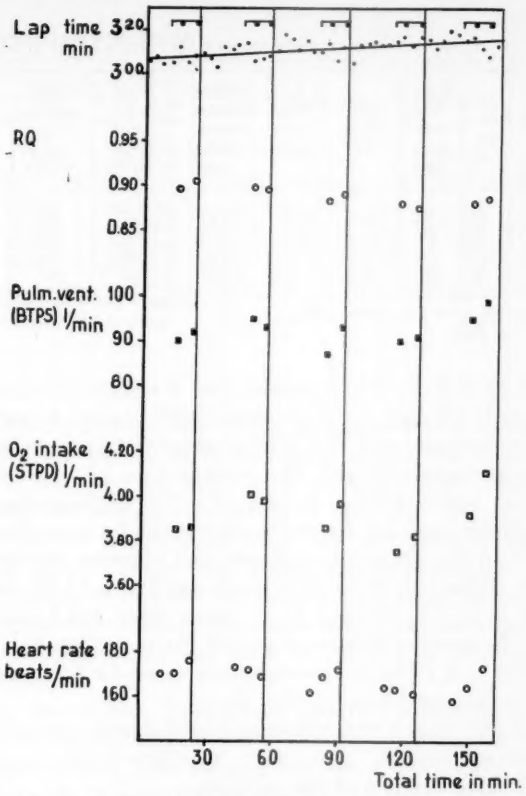


Fig. 2. Subject R. H. Heart rate (○), oxygen intake (□), pulmonary ventilation (■), RQ (○) and lap times ( • ) at ski running 35 km (47 × 750 m) while fasting. Effective work-time 150.1 min. ⌜⌝ = Breathing through valve. ■ = Collection of expired air.

82 % of their aerobic capacity was utilized. S. M., who ran fastest, used 3.8 l $O_2$/min (78 % of his maximal capacity), and A. K., 3.6 l $O_2$/min (84 % of his maximum). The blood lactic acid concentration three min after completion of the work was never greater than 20 mg %. This is striking, in view of the very high work intensities and the degree of exhaustion.

A closer analysis of the experiments was made in the following way. Fig. 2 shows graphically how the time for one lap varied for

Table

*Summary of the results of the experiments*

| Date 1954 | Subject | Effective work time min | Distance km | Mean speed km/hr | Mean pulse rate beats/min | Mean pulm. vent. l/min BTPS | Mean oxygen intake l/min STPD | % of aerobic capacity |
|---|---|---|---|---|---|---|---|---|
| 9/2 .... | M. C. | 154 | 35.25 | 13.7 | 180 | 87 | 3.9 | 82 |
| 3/2 .... | R. H. | 150 | 35.35 | 14.1 | 170 | 93 | 3.9 | 82 |
| 4/2 .... | A. K. | 119 | 27.75 | 14.0 | 175 | 92 | 3.6 | 84 |
| 10/2 .... | S. M. | 161 | 42.00 | 15.7 | 170 | 89 | 3.8 | 78 |

[1] Calculated as a non-protein RQ.
a Normal at rest.

the 47 laps of R. H. The regression line is statistically calculated according to the formula of FISCHER (1946). As can be seen from fig. 2 the running speed was reduced somewhat during the course of the experimental period. The average time for one lap was 3 min 11 sec, with S. D. of $\pm$ 3.39 sec, or 1.77 % of the average speed ($\bar{y}$). A similar tendency for an increase in the lap time during the last part of the ski experiment was also observed for the other subjects. Subject A. K.'s mean speed was 3 min 13 sec, range $\pm$ 3.36 sec or 1.74 %. Subject M. C., whose speed was 3 min 17 sec, showed the greatest scattering around the regression line, $\pm$ 4.71 sec or 2.39 % of $\bar{y}$ (fig. 3). Corresponding values for S. M. was 2 min 53 sec, $\pm$ 3.50 sec, 2.02 % (fig. 4).

Fig. 2 also shows the values obtained during work for pulse rate, oxygen intake, pulmonary ventilation and respiratory quotient (RQ) for one of the subjects.

*Blood sugar.* In table 3 figures are given for the blood sugar concentrations at rest before work, immediately after work and the lowest values reached after work. In one subject (A. K.) the blood sugar values before and immediately after ski running were identical (about 100 mg %). In general, however, the blood sugar concentrations of the other subjects were 10—15 mg % lower immediately after work than at rest before ski running. The minimal values, however, were obtained half an hour following work (76—77 mg % for M. C. and S. M., A. K. 88 mg %; concerning R. H. see table 2).

*Weight loss and water supply.* S. M. lost 3.0 kg in body weight, R. H. 1.7 kg; M. C. and A. K. 2.8 and 2.3 kg respectively during the actual skiing.

3.

*at ski running on level ground.*

| Mean RQ[1] | Total energy consumption Cal | Carbohydrates from organism's depot | | Body weight loss kg | Blood sugar concentration mg % | | | Blood lactic acid conc. mg % |
|---|---|---|---|---|---|---|---|---|
| | | Cal | g | | a | b | c | |
| 0.87 | 2,970 | 1,630 | 395 | 2.8 | 103 | 85 | 76 | 13 |
| 0.89 | 2,900 | 1,830 | 445 | 1.7 | 96 | 89 | 76 | 19 |
| 0.89 | 2,080 | 1,300 | 315 | 2.3 | 98 | 102 | 88 | 20 |
| 0.84 | 2,960 | 1,370 | 335 | 3.0 | 89 | 79 | 77 | — |

b Immediately after ski running
c Minimum after ski running

The subjects were allowed to drink as much water as they desired after the work period. R. H. consumed during the afternoon a total of $^1/_2$ liter, and the other subjects drank about 1 liter each. Thirst was not conspicuous during or after ski running; a sensation of hunger on the other hand was very definite. The subject R. H. first felt hunger and fatigue after approximately 23—24 km of running (*i. e.* after 100 min with a total carbohydrate consumption of some 275 g); thereafter fatigue became progressively more pronounced, so that after 35 km he had to discontinue running.

*Energy consumption.* To obtain as accurate a determination as possible of the quantity of oxidized carbohydrates, the experimental period has been divided into 5—6 subsidiary periods (marked in fig. 2 with vertical lines). The mean value for the double determinations of oxygen intake and RQ within each subsidiary period has been used to represent that whole period.

Table 4 shows more in detail the calculations of the carbohydrate amounts which were consumed during the experiment for the times shown in fig. 2. Carbohydrate oxidation was determined from the data of CATHCART and CUTBERTSON (1931).

Table 3 gives a comparison of the values which were obtained from the four subjects. (The columns for pulse rate, pulmonary ventilation, oxygen intake and RQ indicate the mean values for all the determinations.) In calculating the energy expenditure and amount of carbohydrates consumed, the procedure examplified by table 4 was used. (Total number of Cal, Cal and g from carbohydrates have been rounded off to the nearest 5.)

**Table 4.**

*Subject R. H. Determination of carbohydrate consumption at ski running on level ground, 35.25 km. The experiment was performed on a 750 m circular track with the subject fasting and without a supply of carbohydrates or water during ski running.*

| | Total time min | Number of laps 750 m each | Effective work time min | Mean oxygen intake l/min STPD | Mean RQ | Cal/min | Cal/eff. work time | Carbohydrates from organism's depot | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Cal | g | % of total calories |
| | $23^{45}$ | 7 | 21.8 | 3.86 | 0.90 | 19.05 | 414.7 | 273.7 | 66.8 | 66.0 |
| | $57^{35}$ | 10 | 31.5 | 4.00 | 0.90 | 19.74 | 621.4 | 410.1 | 100.0 | 66.0 |
| | $92^{00}$ | 10 | 32.2 | 3.92 | 0.89 | 19.31 | 621.2 | 388.3 | 94.7 | 62.5 |
| | $126^{30}$ | 10 | 32.2 | 3.79 | 0.88 | 18.62 | 600.1 | 354.1 | 86.4 | 59.0 |
| | $162^{20}$ | 10 | 32.4 | 4.02 | 0.89 | 19.80 | 642.1 | 401.3 | 97.9 | 62.5 |
| Sum: | $162^{20}$ | 47 | 150.1 | — | — | — | 2,899.5 | 1,827.5 | 445.8 | — |

Total energy consumption during the work period for S. M. represented 2,960 Cal and for M. C. 2,970 Cal. For S. M. 46 % of those expended calories originated from the body carbohydrates, or 335 g. In comparison, the figure for M. C. is 55 %, or 395 g. R. H. consumed a total of 2,900 Cal and A. K. 2,080 Cal. These subjects had a mean RQ of 0.89. The consumption of carbohydrates amounted to 445 g for R. H. and for A. K. only to 315 g due to the shorter running time.

*Urine analyses.* In table 2 also the figures for R. H. for the urine nitrogen content are shown, which, after a "normal day" with food intake and without work amounted to 19.16 g (8.94 in the day urine and 10.22 g in the night urine). During the day of the experiment the amount was 11.98 g (5.38 g and 6.60 g respectively). M. C. had a total of 15.22 g and S. M. 9.94 g nitrogen in the urine from the day of the experiment. Complete information as to the urinary nitrogen output during their "normal day" is not to hand.

Subject A. K. had no acetonuria on any occasion. S. M. and R. H. were negative immediately after the ski running, but showed traces of acetonuria during the subsequent period. Only one subject (M. C.) showed gross amounts of acetone in the urine collected immediately after the end of ski running. R. H., M. C. and S. M. showed traces of albumen in the urine collected shortly after ski running. A. K. had, on the other hand, no proteinuria.

### (B) Rest and Standard Work.

*Rest.* Finally, table 5 shows a comparison of the pulse rate, oxygen intake and RQ determined under basal conditions on a "normal day" and 6 to 7 hours after 30 to 40 km of ski running. All the subjects had a very low pulse rate in the basal state, 43—51 beats/min. The oxygen intake was in mean 0.29 (0.27—0.31) l/min and a mean RQ at rest of 0.84 (0.82—0.87) was found. The high RQ for A. K. certainly depended upon hyperventilation, for which reason the estimated RQ in all probability did not correspond with the real value and has been excluded from the mean.

The determinations made 6 to 7 hours after ski running shows on the whole the normal rest values for heart rate, in mean 48 (46—50) beats/min, and oxygen intake, in mean 0.31 (0.27—0.36) l/min. The RQ values were extremely low, particularly for R. H., who had consumed the largest amount of carbohydrates. An RQ of 0.71 measured 6 ½ hours after ski running indicates a practically pure fat oxidation.

*Standard work.* Table 5 also shows that the standard work of 600 kpm/min on the bicycle ergometer, which was performed 3 ½—6 ½ hours after ski running, resulted in an increase in the pulse rate by 10—14 beats/min above the normal work values, which were 88—96 beats/min. Unfortunately no determinations were made for M. C. during a "normal day", but the high work pulse of 123 beats/min also indicates an increase in his case. The oxygen intake during the test work moreover was higher than normal. The RQ obtained during work is extremely low. R. H. and M. C. obtained only 5 % of their caloric supplies from carbohydrates, against the normal 50 %. (RQ = 0.73, against the normal 0.86.) Both subjects suffered from headaches during the test work. S. M. and A. K., on the other hand, obtained 20 to 25 % of their calories from carbohydrates and did not suffer cephalic symptoms.

## Discussion.

Christensen and Hansen (1939), among others, have shown, that the respiratory quotient during work besides being dependent on the food content of fat and carbohydrates, also depends on the work intensity; that is the size of oxygen intake along with the individual's capacity to satisfy the oxygen

Table

*Comparison of pulse rate, oxygen intake and RQ at rest and during work running. The determinations were*

| Date 1954 | Subject | Before ski running | | | After | | | |
|---|---|---|---|---|---|---|---|---|
| | | Basal conditions | | | Rest conditions | | | |
| | | Pulse rate | $O_2$ intake l/min STPD | RQ | Number of hours | Pulse rate | $O_2$ intake l/min STPD | RQ |
| 9/2 ..... | M. C. | 51 | 0.31 | 0.87 | 7 | 48 | 0.36 | 0.75 |
| 3/2 ..... | R. H. | 45 | 0.28 | 0.84 | 6 1/2 | 46 | 0.29 | 0.71 |
| 4/2 ..... | A. K. | 46 | 0.29 | (0.98)[1] | 6 | 48 | 0.27 | (0.92)[1] |
| 10/2 ..... | S. M. | 43 | 0.27 | 0.82 | 5 1/2 | 50 | 0.30 | 0.75 |

[1] Subject hyperventilated.

need. During relatively light and medium heavy work the same RQ remains, similar to that at rest, but during severe work RQ increases considerably.

Moreover CHRISTENSEN and HANSEN found, in agreement with DOUGLAS and COURTICE (1936), that the RQ during prolonged work clearly showed a tendency to decrease during the course of the work and presumed this to be due to the progressive drop in the glycogen reserves.

The results reported here do not agree with the above, as our subjects show very small variations in RQ, which may be due to our high work intensity. As can be seen from fig. 2 the RQ of R. H. was practically constant during the entire run. His first work-RQ was 0.90, and after 150 minutes of ski running, when exhaustion forced him to stop and he had oxidized 445 g of glycogen, the RQ was 0.89, which suggests that 60 % of the energy was still obtained from carbohydrate sources (oxygen intake 4.0 l/min). It seems as if the RQ remains high as long as the work intensity is high. Even if CHRISTENSEN and HANSEN found an RQ-decrease at prolonged work, the RQ was high, although 400 g of carbohydrates had been oxidized. When, however, the organism's glycogen depots are reduced too much, the subject is obliged to decrease his work intensity or, if he has a strong will, he can work with the same high load until he collapses.

For R. H. the first determination in a resting state after ski running gave an RQ = 0.75, and during the afternoon, when the subject was occupied with varied writing tasks, the RQ decreased

5.

*on the cycle ergometer during a "normal day" and after 30—40 km of ski made during the 6th—8th min of work.*

| ski running | | | | "Normal day" | | |
|---|---|---|---|---|---|---|
| Work of 600 kpm/min | | | | Work of 600 kpm/min | | |
| Number of hours | Pulse rate | $O_2$ intake l/min STPD | RQ | Pulse rate | $O_2$ intake l/min STPD | RQ |
| 4 1/2 | 123 | — | 0.73 | — | — | — |
| 3 1/2 | 110 | 1.60 | 0.73 | 96 | 1.49 | 0.86 |
| 6 1/2 | 104 | 1.62 | 0.78 | 94 | 1.61 | 0.82 |
| 4 | 98 | 1.69 | 0.77 | 88 | 1.55 | 0.91 |

further (table 2), and 6 1/2 hours after ski running reached a value of 0.71. The presence of acetone in the urine 2 to 19 hours after the skiing substantiates this high fat oxidation.

The fatigue and headache which caused the subject R. H. to complain, were probably a manifestation of hypoglycemic symptoms. The blood sugar concentration which immediately after ski running was 89 mg %, reached after the cycle test a minimal value of 76 mg % (CHRISTENSEN and HANSEN also noted in their experiments blood sugar values of 60—80 mg % with hypoglycemic symptoms). All tasks in the afternoon were performed with the utmost dislike and the last work test of the day was, in spite of the relatively low intensity, accomplished with difficulty. From the work test, which was made in the morning after the ski experiment and with the subject continuing fasting, higher RQ and blood sugar values were obtained than mentioned above (0.75 and 80 mg % respectively). The reason might be that proteins were transformed to carbohydrates. On the evening of 3/2, as shown in table 2, the RQ at rest was 0.71 and in following morning, 0.74. The protein metabolism during this time rose to 41.25 g. According to LUSK (cited by BEST and TAYLOR 1955) "the quantity of aminoacids present in 100 g of protein is sufficient to form some 58 g of glucose". The converted proteins should consequently be able to give 98 Cal of carbohydrates. With a basal metabolism corresponding to 0.29 l $O_2$/min and an RQ of 0.74 the carbohydrate consumption during the night may have corresponded to 82 Cal. During the 8 minutes of work, when the RQ increased to only 0.75, about 7 Cal of carbohydrates were

consumed. The total carbohydrate consumption between the two RQ determinations can at the maximum have corresponded to 89 Cal. That indicates that the protein conversion was of such magnitude that the oxidized carbohydrates could have originated from proteins.

During a "normal day" R. H. oxidized 5.0 g of protein per hour. During the day of the experiment, when no proteins were supplied, the protein expenditure was lower or 3.1 g per hour.

The RQ of subject A. K. was 0.93 (oxygen intake 3.86 l/min) during the first 21 min of work. During the following 98 min of ski running the RQ was constant (= 0.87). (The oxygen intake decreased from 3.55 to 3.37 l/min.) A. K. never displayed any subjective or objective hypoglycemic symptoms. There was no reduction of the blood sugar concentration during the ski running. No acetone ever appeared in his urine. The RQ of 0.78 during his work test after ski running also indicated that his glycogen depots were not depleted.

Even the subject M. C. had a fairly constant RQ (0.90—0.86) during the first 147 min of actual ski running, when he was able to maintain a constant work intensity (oxygen intake 3.9—4.0 l/min). After this his glycogen depots were reduced to a considerable extent (385 g); this may explain why he was obliged to decrease his work intensity (oxygen intake 3.3—3.6 l/min). The RQ decreased, but as the work intensity was still high, the RQ remained at a high level (0.81—0.82), in spite of the presence of hypoglycemic symptoms. The blood sugar concentration immediately after work was 85 mg % and 30 min later 76 mg %. The large amount of acetone, which appeared in the urine shortly after the ski running, the RQ of 0.73, obtained in the work test 4 $^1/_2$ hours after ski running, and indeed the headache during this last work test, gave evidences of a condition with hypoglycemic symptoms.

During the first work period the RQ for subject S. M. was 0.88, but during the following 140 min of actual ski running the RQ remained at a constant level of 0.84—0.82, which agrees with the results for the other subjects. His blood sugar concentration of 77 mg % and the presence of acetone in the urine indicate hypoglycemic symptoms. But as the total amount of oxidized carbohydrates was only 335 g, this subject presumably had not stored glycogen in the same degree as the others.

The noted relatively low glycogen metabolism with the élite trained S. M. is striking. Only 46 % of the calories were supplied by carbohydrates at an oxygen intake of 53 cc per min and per kg body weight, while at the same oxygen intake R. H. and A. K. mobilized 63 % of the total calories from glycogen. This fully agrees with what CHRISTENSEN and HANSEN found: the RQ is dependent upon the subject's physical fitness. At a certain work level the untrained subjects have a higher RQ than the trained.

The mechanical efficiency is diminished by pure fat oxidation. A comparison of the oxygen intake in our work tests before and after ski running (table 5) emphasizes what KROGH and LINDHARD (1920) have earlier found, namely that a transition from carbohydrate to fat metabolism reduces the mechanical efficiency by 5—10 %. The increase of the pulse rate by 10—14 beats/min during the standard work after ski running certainly depends partially upon a higher oxygen expenditure than under normal conditions (influence of this can be calculated to 2—4 beats/min), but other causes, such as dehydration also have contributed. The liquid deficit was 1—2 1/2 liters after ski running. However, no great thirst was felt. Only 1/2—1 liter of water was taken in during the afternoon. It has also been noted in earlier investigations that sensations of thirst do not correspond to the need for liquid (ADOLPH 1947).

## Summary.

The type of work was ski running at a constant speed on a 750 m circular track set up on level ground. Four well trained male subjects performed this work to the point of exhaustion. During 150—160 min three of the subjects were able to run at a work intensity which corresponded to an oxygen intake of 3.6—4.1 l/min. (The oxygen intake for the fourth subject was 3.4—3.9 l/min during 120 min.) The total energy expenditure during this time was 2,000—3,000 Cal; the pulmonary ventilation rose to a mean of 90 l/min and the heart rate was 160—190 beats/min. By determination of the respiratory metabolism the quantity of oxidized carbohydrates was calculated.

The RQ values remained during the whole run at a practically constant level. One subject, who had reduced his glycogen depot by 445 g, continued to get 55 % of his calorie need from carbohydrates, although hypoglycemic symptoms were present.

The blood lactic acid concentrations at the end of these long periods of work were slightly increased (20 mg %).

## Supplement.

The results of the questions on food, liquid supply *etc.*, discussed above, may directly be applied to ski competitions of 20 km and longer. Accordingly, certain determinations were made on the subject S. M. in 1954 during the classic Vasa race, the marathon of skiing, 85 km. (S. M. came in 6th among 580 competitors.) The heart rate during ski running was determined by taking the time for 10 pulse beats measured immediately after the subject had stopped for food and water intake, the amounts of which were also noted. These determinations were made after 24, 32, 46, 58, 70, 81 and 85 km of ski running. The stop at each of the nine control stations took about half a min, why his effective work time was 6.68 hours and his mean speed 12.8 km/hour. The track was in a hilly terrain. During that time his pulse rate ranged between 150—180 beats/min, which offers some conception of the work intensity. His rectal temperature was 36.6° C before ski running and 38.4° C after the race. The subject took in as much fluid as he desired, and the total liquid consumption during the race was 1.4 liters. In spite of that the weight loss was 4.6 kg, which indicates a heavy dehydration. The mean pulse rate for the seven determinations mentioned above was 160 beats/min. According to table 3 this subject had in the ski experiment of the 10th of Febr. an oxygen intake of 3.8 l/min (mean value of 12 determinations) at a heart rate of 170 beats/min. Fig. 4 gives at pulse rate 160 beats/min an oxygen intake of about 3.6 l/min. The mean RQ was 0.84. Accepting these values as a basis, the RQ during the Vasa run, on the average, was 0.83—0.84. If this assumption is correct, the total energy output during his ski running was approximately 7,000 Cal and about 710—770 g of carbohydrates were oxidized. At the control stations, in accordance with the above and also after 63 and 78 km of ski running, the subject got small amounts of carbohydrates in the form of sugar water and very sweet whortleberry sauce in order to keep his blood sugar concentration at a sufficient level so that the competition could be completed. The above given quantity of fluid of 1.4 liters contained 370 g of carbohydrates. The organism's own glycogen depot would, therefore, have contributed 340—400 g.

The RQ determination at rest 2 hours after completion of ski running showed pure fat oxidation (RQ = 0.70). The expired air smelled of acetone. (The conditions of the experiment did not allow any determination of the blood sugar concentration, but in spite of that one may venture to assert that exhaustion towards the end of running was due to hypoglycemia.)

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# A Modification of the Thiocholine Method for the Determination of Cholinesterase.

## I. Biochemical Evaluation of Selective Inhibitors.

By

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Among the various methods available for the histochemical localization of esterases (RAVIN, TSOU and SELIGMAN 1951, HOLT 1954), the thiocholine method (KOELLE 1950, 1951*a*, *b*, *c*, 1955) is the only one which is reasonably specific for cholinesterases and which permits the separate identification of the two main types of this enzyme found in the animal body. Briefly, the thiocholine method depends upon the enzymic hydrolysis of carboxylic acid esters of thiocholine sulphate in the presence of a cupric ion complex, followed by precipitation of the liberated thiocholine sulphate as the copper mercaptide which, according to MALMGREN and SYLVÉN (1955), has the structure

$$Cu^{+} \cdot S \cdot CH_2CH_2N^{+}Me_3,\ SO_4^{--}.$$

Finally, the precipitated copper thiocholine is converted into copper sulphide at the site of deposition by treatment with ammonium sulphide. In the absence of a selective substrate for acetocholinesterase (AcChE) (= true-, specific cholinesterase, ChE I of other authors), the method is made specific for this enzyme by using acetylthiocholine (AcThCh) as substrate in the presence of a selective inhibitor of butyrocholinesterase (BuChE) (= pseudo-, non-specific cholinesterase, ChE II). It is made specific for BuChE either (a) by the use of butyrylthiocholine (BuThCh) as substrate or (b) by the use of a selective inhibitor of AcChE with AcThCh as substrate. With AcThCh as substrate

and no selective inhibitor, both cholinesterases are revealed simultaneously. To control the possible hydrolysis of thiocholine esters by thiolesterases distinct from cholinesterases, it is advisable to include tissue sections which have been treated (a) with both selective inhibitors followed by AcThCh (b) with the BuChE inhibitor followed by BuThCh. These controls will be blank if thiolesterase is absent.

It will thus be appreciated that the success of the method in distinguishing between the two types of cholinesterase depends very much on the selectivity of the inhibitors used. Di*iso*propoxyphosphoryl fluoride (DFP) (for nomenclature of organophosphorus compounds see LARSSON, HOLMSTEDT and TJUS 1954) has most often been used as the selective BuChE inhibitor, but a survey of the literature shows an astonishing variation (amounting to over three powers of ten) in the concentrations used and the type of biochemical control applied. Previous investigators, when they have carried out controls at all, have usually been content to perform these manometrically at a different pH and under different conditions of concentration and composition of reaction medium from those actually prevailing in the histochemical studies. Yet inhibition of cholinesterases is known to be markedly affected by pH (HEILBRONN 1954). The use of organophosphorus compounds is also complicated by the presence in many tissues of the so called DFP-ases (MOUNTER, DIEN and CHANUTIN 1955) or phosphorylphosphatases (AUGUSTINSSON 1954) which hydrolyse these inhibitors and possibly the inhibited enzyme complex.

As a preliminary to a programme of histochemical work with cat tissues a number of selective cholinesterase inhibitors have been evaluated for their possible use in the thiocholine method under conditions closely similar to those used histochemically. This has been made possible by the electrometric method developed by TAMMELIN (1951, 1952, 1953) which is as accurate as the manometric method and has the advantage that it can be used at any pH. In the present investigation all the chemicals used by KOELLE histochemically have also been present in the biochemical experiments. The only alteration is that the buffer has been diluted four times to decrease the capacity and make electrometric determinations possible. The study has been carried out using purified cholinesterases representative of the two main types and also crude preparations of these enzymes from the cat.

## Materials and Methods.

### *Enzymes.*

*Electric organ AcChE.* Freeze dried tissue of electric organ of *Torpedo marmorata* (500 mg) was freshly prepared for each experiment by homogenizing in water at 12,000 rpm for 10 min. and diluting to 100 ml.

*Human serum BuChE.* Cohn's fraction IV-6 (50 mg) in 20 ml. Stable at 0° for 3 days. The fraction was prepared by AB Kabi, Stockholm.

*Cat brain AcChE.* Cat caudate nucleus (1.2 g) was homogenized in 15 ml of water at 12,000 rpm for 10 min. and used immediately.

*Cat serum BuChE.* Cat serum was freshly prepared and used undiluted.

### *Inhibitors.*

Besides DFP the following inhibitors were used: dimethobromide of 1 : 5-bis (*p*-N-allyl-N-methylaminophenyl)pentan-3-one (BW 284c51) (BW), a reversible inhibitor of AcChE (FULTON 1954, AUSTIN and BERRY 1953, DENZ 1954, KOELLE 1955); diethiodides of bis (diethylaminoethyl) terephthalate (302 IS) and bis (diethylaminoethyl) phthalate (306 IS), selective inhibitors of AcChE and BuChE respectively (BOVET-NITTI and BOVET 1953); bismonoisopropylamido-phosphoryl fluoride (Mipafox) a selective inhibitor of BuChE (DAVISON 1953, DENZ 1954).

BW was kindly supplied by the Dept. of Organic Chemistry, Research Institute of National Defence, Sundbyberg 4, Sweden, 302 IS and 306 IS by Prof. D. BOVET, Rome.

Mipafox was synthesized according to the reaction:
$Cl_2PO \cdot F + 4\, i\, C_3H_7NH_2 \rightarrow (i\, C_3H_7NH)_2PO \cdot F + 2\, i\, C_3H_7NH_2 \cdot HCl$,
by adding isopropylamine (0.8 mol.) in 50 ml dry ether during 30 min. to dichlorophosphoryl fluoride (BOOTH and DUTTON 1939) in 100 ml dry ether with vigorous stirring at — 5 to 0°. The reaction mixture was allowed to come to room temperature during 1 hr., filtered and the ether removed *in vacuo*. The residue was distilled *in vacuo*, yielding 22 g (60 %) of the desired product, b.p., 125°/1.5 mm, 116°/0.7 mm, m.p. 60°, $d^{25°}$, 1.2. (Found: N, 15.0, Calc. for $C_6H_{16}N_2POF$ : N, 15.4%).

### *Solutions.*

*Stock solutions.* The following aqueous stock solutions were stored at room temperature unless otherwise stated.

1) Copper glycinate. Glycine (3.75 g) + $CuSO_4 \cdot 5H_2O$ (2.5 g) in 100 ml.

2) Maleate buffer. Sodium hydrogen maleate (9.6 g) + N NaOH (52.2 ml) in 100 ml.

3) 40 % w/v $Na_2SO_4$ adjusted to pH 6 and stored at 38°.

4) 9.25 % w/v $MgCl_2$.

5a) Incubation solution for AcChE. 5 ml 1) + 12.5 ml 2) + 300 ml 3) + 20 ml 4). Kept in sealed bottles at 38°.

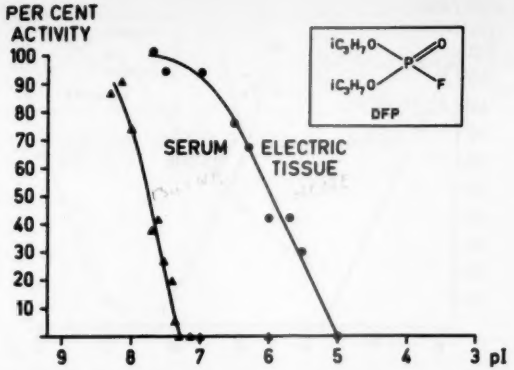


Fig. 1. Inhibition curves for acetocholinesterase (electric tissue) and butyrocholinesterase (serum). Substrate acetylthiocholine iodide. Inhibitor DFP. In this and the following figures circles denote acetocholinesterase. Empty triangles denote the use of butyrylthiocholine iodide as substrate.

5b) Incubation solution for BuChE. 5 ml 1) + 12.5 ml 2) + 350 ml 3) + 20 ml 4). Kept in sealed bottles at 38°.

6) AcThCh iodide (57.5 mg/4 ml), freshly prepared.

7) BuThCh iodide (62.5 mg/ 4 ml), freshly prepared.

8) Inhibitors were made up in appropriate concentrations in solutions 5a) or 5b).

*Reaction mixtures.* Reaction mixtures differing in sodium sulphate concentration and corresponding to the two incubation media used by KOELLE were used for experiments with AcThCh and BuThCh respectively. These contained final concentrations of $Mg^{++}$, 0.04 M, glycine, 0.0005 M, $Cu^{++}$, 0.0011 M, maleate buffer, 0.167 M and substrate, 0.004 M and were made up from the stock solutions as follows:

A) For AcThCh: 2.915 ml 5a) + 0.400 ml 6) + 0.460 ml 8) + enzyme (0.700 ml) + water (0.525 ml) to 5 ml.

B) For BuThCh: 3.415 ml 5b) + 0.400 ml 7) + 0.460 ml 8) + enzyme (0.700 ml) + water (0.025 ml) to 5 ml.

### *Measurement of Enzyme Activity.*

Enzyme activity was determined at 38° and pH 6 electrometrically and is expressed as $\mu$moles acid liberated/ml reaction mixture/min. The apparatus was calibrated by introducing known amounts of acetic acid into the reaction vessel instead of substrate by means of an Agla micrometer syringe (the pK's of acetic and butyric acid are sufficiently close for this calibration to be valid for both substrates). Spontaneous hydrolysis of substrates and inhibitors and the enzymic hydrolysis of 306 IS and 302 IS (BOVET-NITTI and BOVET 1953) were corrected for in control tubes from which enzyme or substrate had been omitted. These corrections never exceeded 2 %.

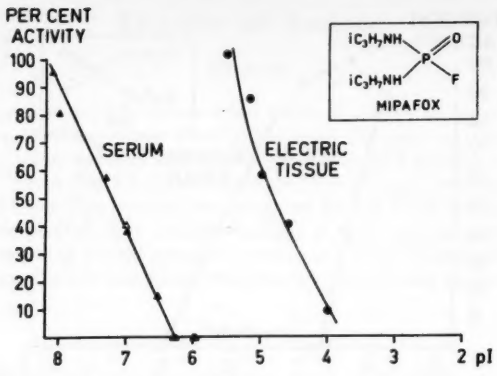


Fig. 2.
Explanation see Fig. 1.

## Results.

### *Inhibition Studies with Purified Enzymes.*

Figs. 1—5 show the effect of the five inhibitors on human serum and electric tissue cholinesterases. Enzyme activity, expressed as a percentage of the activity of the uninhibited enzyme is plotted against the negative logarithm of the inhibitor concentration (pI). A good selective inhibitor for histochemical purposes is clearly one which inhibits one cholinesterase completely at a concentration which leaves the other cholinesterase unaffected. It will be seen (Fig. 1) that DFP is not a particularly good selective inhibitor of BuChE, since it is not possible to select a concentration which gives complete inhibition of BuChE without affecting AcChE. The results show that the original DFP concentration used by KOELLE ($10^{-6}$ M) while sufficient to inhibit BuChE completely, is appreciably inhibitory (ca. 50 %) to AcChE and that his latest concentration ($10^{-8}$ M) is inadequate to inhibit BuChE. Mipafox (Fig. 2) is on the other hand an excellent selective inhibitor for BuChE since there is a ten-fold safety factor between the concentration producing complete inhibition of BuChE ($4 \times 10^{-6}$ M) and that required for minimal inhibition of AcChE. A concentration of $4 \times 10^{-6}$ M Mipafox has therefore been adopted for selective inhibition of BuChE in the histochemical work described in the following paper. This is the same concentration as used by DAVISON (1953) but higher than that used by DENZ

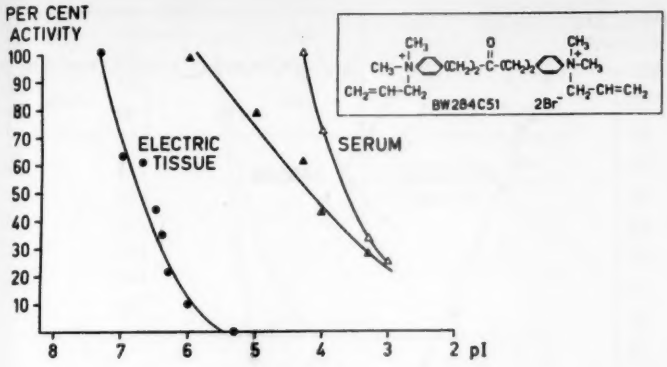


Fig. 3.
Explanation see Fig. 1.

(1954) ($10^{-7}$ M). DENZ apparently did not do control studies, and it is doubtful whether BuChE was more than 60 % inhibited under his conditions.

Fig. 3 shows the results obtained with BW. As would be expected with a competitive inhibitor, the curve of inhibition obtained is dependent on the substrate, as is seen with AcThCh and BuThCh and the serum enzyme. A concentration of $5 \times 10^{-6}$ M has been selected in the present investigations. DENZ (1954) used a concentration of $3 \times 10^{-4}$ M for selective inhibition of AcChE which, according to Fig. 3 gives appreciable inhibition of BuChE.

Fig. 4 shows that 302 IS is also a selective inhibitor for AcChE, only slightly inferior to BW, $10^{-6}$ M being a suitable concentration. 306 IS (Fig. 5), an isomer of 302 IS, is remarkable for the almost vertical inhibition curve with BuChE when AcThCh is the substrate. The degree of inhibition of BuChE is markedly dependent on the substrate. Although selective for BuChE, it is not as good as the other inhibitors tested and was not used further.

*Studies with unpurified cat enzymes.* It was felt essential to check the results obtained with purified cholinesterases against crude preparations from the cat, for the following reasons. Species variations in the sensitivity of cholinesterases to inhibitors have been reported (cf. AUSTIN and BERRY 1953, ALDRIDGE 1953) and there is evidence (COHEN and WARRINGA 1953) that anti-cholinesterases can combine with proteins other than cholinesterases in crude enzyme preparations, thus reducing their availability

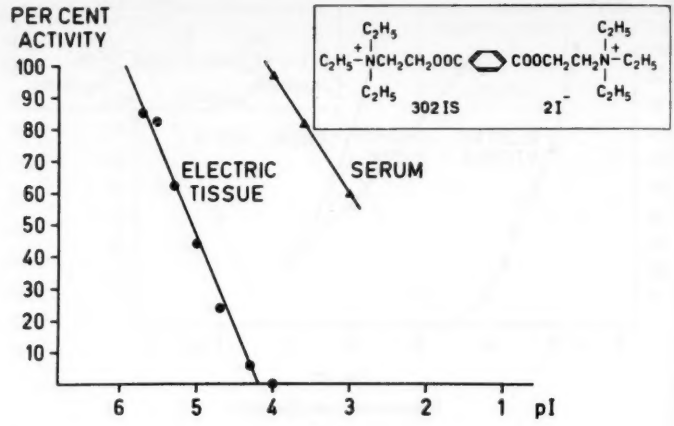


Fig. 4.
Explanation see Fig. 1.

for cholinesterase inhibition. There is also the factor of enzymic destruction of anti-cholinesterases referred to earlier.

Table 1 shows experiments of this kind using the two inhibitors BW and Mipafox. It will be seen that $5 \times 10^{-6}$ M BW, the concentration selectively inhibitory for purified AcChE, abolishes only about 77 % of AcThCh hydrolysis by the cat brain preparation; however, increasing the concentration to $10^{-5}$ M brings about little further inhibition, and $10^{-4}$ M Mipafox, which caused complete inhibition of purified BuChE but no inhibition of purified AcChE (Fig. 2), produced 28 % inhibition of AcThCh hydrolysis by the cat brain preparation. Both inhibitors together abolished AcThCh hydrolysis. It is concluded that the residual, Mipafox sensitive hydrolysis of AcThCh occurring in the presence of $5 \times 10^{-6}$ M BW is due to BuChE which is known to be present in brain tissue (ALDRIDGE 1953, DAVISON 1953). The 2 % additional inhibition of AcThCh hydrolysis occurring when the concentration of BW is raised from $5 \times 10^{-6}$ M to $10^{-5}$ M is probably due to inhibition of BuChE rather than AcChE since BW showed some inhibition of purified BuChE (Fig. 3) at this concentration. The results with the crude brain preparation are thus consistent with selective inhibition of the two cholinesterases by BW and Mipafox at the concentrations deemed selective for the experiments with purified enzymes. Similarly, the cat serum cholin-

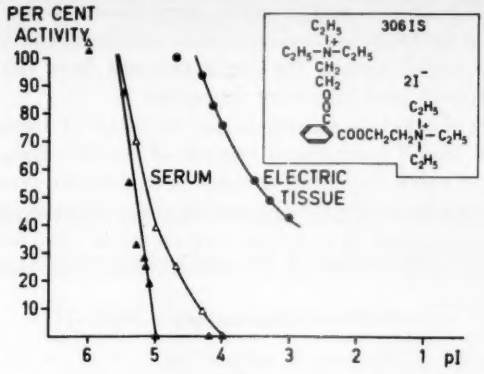


Fig. 5.

Explanation see Fig. 1.

esterase, containing predominantly BuChE (MENDEL, MUNDELL and RUDNEY 1943, AUGUSTINSSON 1948) was completely inhibited by $10^{-6}$ M Mipafox.

**Table 1.**

| Source of enzyme | Substrate | Mol. concentration of inhibitor = I | Neg. log mol. conc. of I = pI | $\mu$M HAc $\times 10^3$ $ml^{-1}min^{-1}$ | Per cent inhibition of enzyme activity |
|---|---|---|---|---|---|
| Cat's brain | AThCh | — | — | 25.7 | — |
| " | AThCh | $4\times10^{-7}$ BW 284c51 | 6.4 | 12.6 | 51 |
| " | AThCh | $5\times10^{-6}$ BW 284c51 | 5.3 | 5.8 | 77 |
| " | AThCh | $10^{-5}$ BW 284c51 | 5.0 | 5.5 | 79 |
| " | AThCh | $10^{-6}$ Mipafox | 6.0 | 18.6 | 28 |
| " | AThCh | $10^{-6}$ Mipafox + $5\times10^{-6}$ BW 284c51 | 6.0<br>5.3 | 0 | 100 |
| Cat's serum | AThCh | — | — | 36.8 | — |
| | AThCh | $10^{-6}$ Mipafox | 6.0 | 0 | 100 |

## Summary.

Biochemical controls of the thiocholine method for the determination of cholinesterase have been performed both with partially purified enzyme and with enzymes from the cat.

The electrometric method that has been used for the determinations permits the use of the same pH and the presence of all the reagents used histochemically. The inhibition curves of aceto- and butyrocholinesterase activity using DFP, Mipafox, BW 284c51, 302 IS and 306 IS have been determined. Of these in-

hibitors BW 284c51 and Mipafox were shown to be the most selective in the histochemical incubation solutions. The compounds were also tested against the crude enzymes from the cat, and showed equally good inhibitory properties.

The use of selective substrates and inhibitors is discussed. It is concluded that if biochemical controls of the thiocholine method are to be of value they must be made in exactly the same solution as used histochemically. The pH and the concentration of inhibitor in the solution must be based on controls run in vitro or erroneous localisation of the two types of cholinesterases may result.

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# A Modification of the Thiocholine Method for the Determination of Cholinesterase.

## II. Histochemical Application.

By

BO HOLMSTEDT.

Received 18 April 1957.

In this paper a modification of the KOELLE histochemical method for cholinesterase is described, using the inhibitors selected as described in the preceding paper. The work has been confined to cat tissues; detailed morphological studies have been excluded as these will be presented in later papers.

The principle modifications which have been introduced into the KOELLE technique are: (1) the use of Mipafox (M) and BW 284c51 (BW) as specific inhibitors of butyrylcholinesterase (BuChE) and acetylcholinesterase (AcChE) respectively; (2) the omission of copper thiocholine from the reaction mixture; (3) the omission of the ammonium sulphide stage before fixing and mounting; (4) the inclusion of adequate controls for thiolesterases.

## Experimental.

### Solutions.

*Stock solutions.* The following stock solutions are required:

(1) *N* NaOH
(2) 0.1 *M* $CuSO_4$
(3) 40 % w/v $Na_2SO_4$ adjusted to pH 6.
(4) 0.04 m*M* M (0.73 mg/100 ml). Stable for 3 days in the cold.
(5) 0.5 mM BW (28 mg/100 ml). Indefinitely stable in the cold.
(6) Copper glycinate. Glycine (3.75 g) + $CuSO_4 \cdot 5H_2O$ (2.5 g) in 100 ml.

(7) Maleate buffer, pH 6 (9.6 g sodium hydrogen maleate + 52.2 ml (1) in 100 ml).
(8) 9.52 % w/v $MgCl_2$.
(9) Acetylthiocholine (AcThCh) sulphate (45 mg AcThCh iodide + 0.8 ml (2) + 2.4 ml water; precipitate removed by centrifuging and discarded). Unstable.
(10) Butyrylthiocholine (BuThCh) sulphate (50 mg BuThCh iodide + 0.8 ml (2) + 2.4 ml water; precipitate removed as in (9). Unstable.

Solutions 3, 6, 7, 8 are conveniently stored in an incubator at 38°.

*Storage and incubation solutions.* From the above stock solution, storage and incubation solutions are made up as shown in Table 1.

**Table 1.**

*Composition of storage and incubation solutions.*

| Solution | Amounts (ml) of stock solutions | | | | | | | | Amount of water (ml) |
|---|---|---|---|---|---|---|---|---|---|
| | 3 | 4 | 5 | 6 | 7 | 8 | 9[1] | 10[1] | |
| $St_1$ | 9 | — | — | — | — | — | — | — | 6.0 |
| $St_2$ | 10.5 | — | — | — | — | — | — | — | 4.5 |
| $I_1$ | 9 | 1.5 | — | — | — | — | — | — | 4.5 |
| $I_2$ | 10.5 | 1.5 | — | — | — | — | — | — | 3.0 |
| $I_3$ | 9 | — | 0.15 | — | — | — | — | — | 5.85 |
| $I_4$ | 10.5 | — | 0.15 | — | — | — | — | — | 4.35 |
| $S_1$ | 9 | — | — | 0.6 | 1.5 | 0.6 | 1.2 | — | 2.1 |
| $S_2$ | 9 | — | 0.15 | 0.6 | 1.5 | 0.6 | 1.2 | — | 1.95 |
| $S_3$ | 10.5 | — | — | 0.6 | 1.5 | 0.6 | — | 1.2 | 0.6 |
| $S_4$ | 10.5 | — | 0.15 | 0.6 | 1.5 | 0.6 | — | 1.2 | 0.45 |

[1] Added just before use.

## Histochemical Procedure.

Fresh frozen sections 10—15 $\mu$ thick are mounted directly on slides. Seven slides (denoted by letters A—G) are needed for a single determination of the two cholinesterase types with full controls. They are treated according to the procedure summarized in Table 2.

*Stage 1.* The slides are kept for as short a time as possible in the storage solutions.

*Stages 2—4.* Slides B, C, D, F, G are transferred to the appropriate inhibitor solutions and incubated at 38° for 30 min. The slides treated with M (B, D, F) are washed in fresh solutions of the same composition as their original storage solutions for 10 min. Slide D is then further incubated with fresh BW (solution $I_3$) for 30 min. at 38°.

*Stage 5.* The slides are finally incubated with substrates. The incubation time depends upon the tissue. Slides A and E are transferred directly from their storage solutions, the others, from the inhibitor (C, D, G) or wash (B, F) solutions. Since BW is a reversible inhibitor, slides which have been treated with this inhibitor are incubated with substrate in the presence of the inhibitor.

**Table 2.**

*Summary of histochemical procedure.*

For composition of solutions see Table 1.

| | | | | |
|---|---|---|---|---|
| Stage 1 | Storage | | | |
| Slides | ABCD | | EFG | |
| Solutions | $St_1$ | | $St_2$ | |
| Stage 2 | Incubation with inhibitor (30 min. at 38°) | | | |
| Slides | BD | F | C | G |
| Solutions | $I_1$ | $I_2$ | $I_3$ | $I_4$ |
| Stage 3 | Wash (10 min.) | | | |
| Slides | BD | F | | |
| Solutions | $St_1$ | $St_2$ | | |
| Stage 4 | Reincubation with inhibitor (30 min. at 38°) | | | |
| Slide | D | | | |
| Solution | $I_3$ | | | |
| Stage 5 | Incubation with substrates (5—120 min. at 38°) | | | |
| Slides | AB | CD | EF | G |
| Solutions | $S_1$ | $S_2$ | $S_3$ | $S_4$ |

The slides are viewed without further treatment in the ordinary light microscope and photographed if a permanent record is required.

It will be noted that presaturation of the incubation medium with copper thiocholine and conversion of the copper thiocholine precipitated during the reaction to copper sulphide with ammonium sulphide are omitted. A series of sections from cat striated muscle incubated with and without copper thiocholine showed no difference in the pictures obtained. In another series, treated with ammonium sulphide in varying dilutions and for varying times, some diffusion was always evident. Treatment of the wet slides with gaseous $H_2S$ gave better pictures, but it was found that the precipitated copper thiocholine is itself visible in both the phase contrast and ordinary light microscope, and that conversion to copper sulphide is unnecessary. The pictures included in this paper have been made without ammonium sulphide treatment, contrast staining or gold toning.

### Interpretation of results.

Table 3 summarizes the treatment accorded each slide and the type of cholinesterase which should be visible if the basic assumptions of the method are correct. These are: that neither substrate is attacked by esterases other than cholinesterases; that BuThCh is attacked only by BuChE; that M and BW are selective inhibitors for the two cholinesterases under the conditions used. Figs. 1 and 2 show, that the results obtained with two representative tissues are consistent with these assumptions.

**Table 3.**

*Interpretation of results of histochemical method.*

| Slide | Treatment | | ChE visualized |
|---|---|---|---|
| | Inhibitor | Substrate | |
| A | — | AcThCh | AcChE+BuChE |
| B | M | AcThCh | AcChE |
| C | BW | AcThCh | BuChE |
| D | M + BW | AcThCh | Blank |
| E | — | BuThCh | BuChE |
| F | M | BuThCh | Blank |
| G | BW | BuThCh | BuChE |

Fig. 1 shows results obtained with the stellate ganglion. It will be seen that in accordance with the predictions of Table 3, sections D and F are blank, C, E and G are identical, except for some variation in intensity, and that everything staining in B and C also appears in A.

On slides A and B, three types of ganglion cell can be distinguished, namely those giving intense, faint and no staining for AcChE. These three types have also been observed by KOELLE (1949—55) and isolated by GIACOBINI (1956, 1957). They can always be observed in varying proportions in sections of sympathetic ganglia, but not in other portions of the nervous system (GIACOBINI and HOLMSTEDT 1957). A closer inspection shows that the nuclei are unstained or much less stained than the cytoplasm; some staining occurs with prolonged incubation. This result accords with the findings of GIACOBINI (1957) who has shown by microdissection that the cytoplasm contains much more cholinesterase than the nucleus.

Fig. 2 shows sections of striated muscle. Once again the distribution of blank and stained slides accords with the predictions of Table 3. The interesting result emerges from these slides that the end plates contain both types of cholinesterase. BuChE has not previously been reported in end plates though DENZ (1954), using rat diaphragm, found staining with BuThCh after long incubation (4 hr.).

Fig. 3 demonstrates the distribution of AcChE and BuChE in the fundic region of the cat's stomach wall. Only B and G of the complete series of slides are reproduced. It will be seen that AcChE occurs only in the ganglion cells of the plexus, while

BuChE occurs in the mucosa, the muscularis mucosae outlining the submucous glands and in some ganglion cells as previously reported by KOELLE.

## Discussion.

The slides demonstrate, that the modified KOELLE histochemical method described in this paper can effectively demonstrate the distribution of the two types of cholinesterase in cat tissues. The controls included in the procedure provide a check against errors in the identification of cholinesterase types, at least as important a consideration in any histochemical method for cholinesterase as the elimination of diffusion artefacts.

It is felt, that the omission of copper thiocholine from the incubation solutions and the elimination of the ammonium sulphide step, both features of the KOELLE technique, have if anything improved the degree of localization. Working with blood cells, ZAJICEK, SYLVÉN and DATTA (1954) noted needle-shaped crystals at the site of enzyme activity which gradually increased in size and eventually projected outside the site of enzyme activity if incubation was prolonged. MALMGREN and SYLVÉN (1955), in similar experiments using phase contrast microscopy, found that secondary growth of primary crystals did not occur if copper thiocholine was omitted. During several years work with tissue sections the author has often seen crystals at the site of enzyme activity, especially after prolonged incubation; for this reason, incubation times have been kept as short as possible. Crystals have also been seen by COUTEAUX and TAXI (1952) and MOHR and GEREBTZOFF (1954).

The results of ZAJICEK et al. (1954) and MALMGREN and SYLVÉN (1955) also suggest, that the conversion of copper thiocholine to copper sulphide increases the risk of artefacts. Thus MALMGREN and SYLVÉN (1955) observed microscopically that copper thiocholine crystals dissolved on treatment with ammonium sulphide and were replaced by an amorphous dark brown precipitate. With weaker solutions of ammonium sulphide, the crystals did not disappear, but became coated with a layer of copper sulphide. It is difficult to believe, in the face of this evidence, that the copper sulphide can indicate as accurately as the thiocholine which it replaces the precise location of the enzyme activity.

In the present work, fresh frozen tissues were used in preference to formalin fixed tissues, since as the manometric studies of TAXI (1952) show, formalin is strongly inhibitory to cholinesterases and does not necessarily affect the two types equally. Authors using formalin fixation (COUTEAUX et al. 1952, COËRS 1955, 1956, GEREBTZOFF 1953) have not carried out adequate controls. Thus while the thiocholine method in conjunction with formalin fixation may be useful as a purely histological method, it is at present inadequate for localizing the different types of cholinesterases and for this purpose the advantages of the fresh frozen method outweigh the disadvantages mentioned by PEARSE (1955).

## Summary.

Based upon the biochemical controls of the thiocholine method (HOLMSTEDT 1957) the various steps of the histochemical procedure have been investigated. The inhibitors Mipafox and BW 284c51 and the substrates acetylthiocholine and butyrylthiocholine were combined as shown in Table 3 with the object of producing a reliable method of separating the two types of cholinesterase. Sufficient controls are included in the table to allow a distinct separation of the two types of cholinesterase.

The technique of the modified thiocholine method is given in detail. The following modifications have been made.

1. Inclusion of new inhibitors as above. (Mipafox and BW 284c51.)
2. No presaturation with copper thiocholine.
3. No development with ammonium sulphide.

With the modified method, stellate ganglion, striated muscle and stomach wall have been stained in order to produce confirmation of the biochemical controls, not with the purpose of studying the morphology in detail. Comparison of sympathetic ganglia and motor end-plates shows that the ganglion cells contain only acetylcholinesterase but that the motor end-plates also contain a considerable amount of butyrylcholinesterase.

The reasons for the alterations of the original thiocholine method are discussed. The exclusion of the presaturation with copper thiocholine produces no effect on the staining of tissue sections. The exclusion of development with ammonium sulphide diminishes diffusion artefacts to a considerable degree. Only fresh frozen sections can be used at present, if the purpose is to distinguish between the two types of cholinesterase.

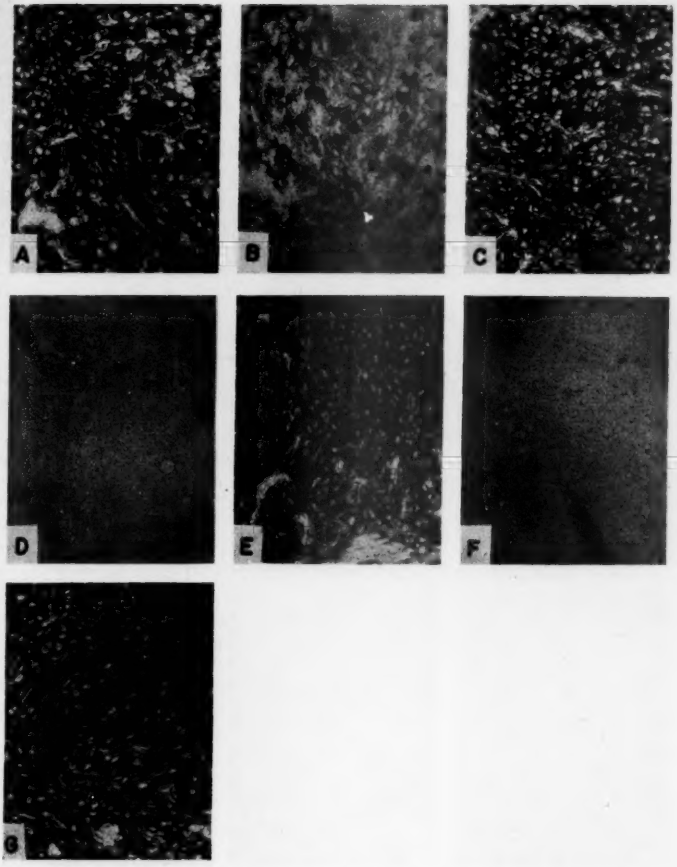


Fig. 1. Stellate ganglion from cat. Modified thiocholine method. Complete series of combinations of substrates and inhibitors. No presaturation with copper thiocholine. Incubation time 30 min. Magnification 96 times. Untouched photography in ordinary light microscope.

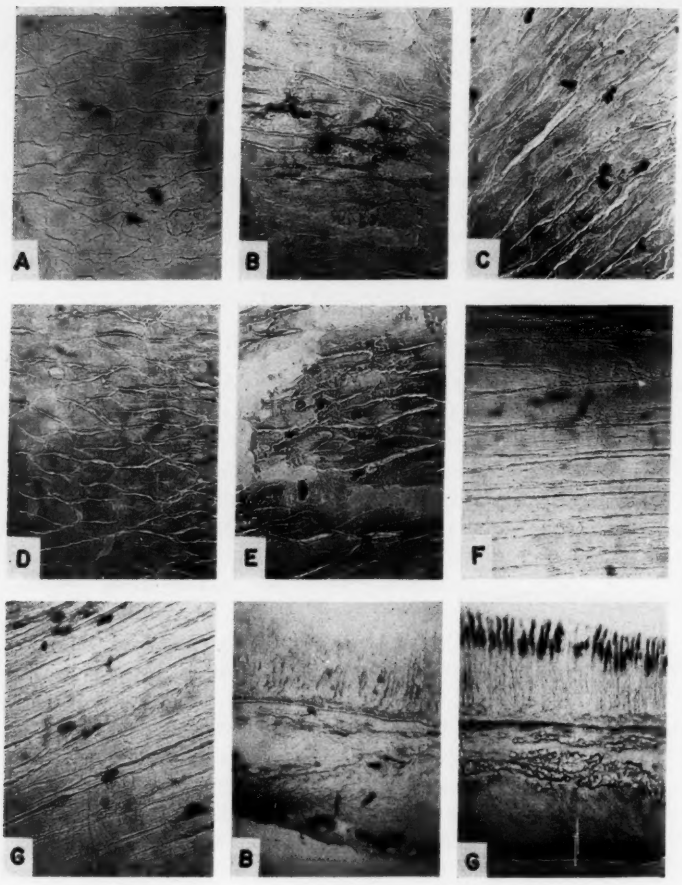


Fig. 2. Fig 3.

Fig. 2. (A - G.) Striated muscle from cat. Modified thiocholine method. Musculus biceps femoris. Magnification 120 times. Treatment the same as for stellate ganglion.

Fig. 3. (B and G lower right.) Stomach wall from cat. Modified thiocholine method. Magnification 46 times. Treatment like stellate ganglion.

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# The Toxicity of Low-molecular Triglycerides.

By

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Received 30 April 1957.

Intravenously administered emulsions of tributyrin have a marked effect in respiration and blood pressure and a surprisingly high toxicity (WRETLIND 1957). On the basis of these observations, the toxicity of other low-molecular triglycerides has been investigated. The $LD_{50}$ of intravenous emulsions of triglycerides with fatty acids containing from 2 to 11 carbon atoms was determined in groups of mice.

## Experimental.

All the triglycerides used were produced synthetically by esterification of glycerin and the relevant fatty acids in the presence of TWITCHELL's reagent (naphthalinsulphostearic acid) according to the method of OZAKI (1926). Triacetin, tripropionin, tributyrin, triisovalerin, tricapronin, triheptylin, tricaprylin and trinonylin were purified by distillation *in vacuo*. Tricaprinin and triundecylin were recrystallized two times from ether-alcohol solution.

Triglycerides containing fatty acids with higher molecular weight were not investigated because stable emulsions could not be produced with the system used.

The various neutral fats were prepared in either 10 or 25 per cent emulsions of the following composition:

| | 10% Emulsion | 25% Emulsion |
|---|---|---|
| Triglyceride | 10 g | 25 g |
| Phosphatides | 0.75 g | 1.88 g |
| Demal 14 | 1 g | 2.5 g |
| Glucose solution, 5% | ad 100 ml | ad 100 ml |

The phosphatides were prepared from soy beans (WRETLIND 1957). Demal 14[1] (polyglycerol monooleate) was used without purification.

[1] Supplied by the Emulsol Corporation, Chicago, Ill.

**Table I.**

*The various triglycerides tested, the number of carbon atoms in their fatty acid, and the $LD_{50}$ with its standard error.*

| Substance | No. of carbon atoms in fatty acid | $LD_{50}$ mg/kg |
|---|---|---|
| Triacetin ..... | 2 | 1,600 ± 81 |
| Tripropionin .... | 3 | 840 ± 34 |
| Tributyrin ..... | 4 | 320 ± 11 |
| Triisovalerin .... | 5 | 82 ± 4 |
| Tricapronin ..... | 6 | 122 ± 5 |
| Triheptylin ..... | 7 | 320 ± 12 |
| Tricaprylin ..... | 8 | 3,700 ± 194 |
| Trinonylin ..... | 9 | > 10,000 |
| Tricaprinin ..... | 10 | > 10,000 |
| Triundecylin .... | 11 | > 10,000 |

The emulsions were prepared and homogenized in a Logeman homogenizer as described earlier (WRETLIND 1957).

Determination of $LD_{50}$ was performed on mice weighing from 13 to 29 g. The emulsion was injected into a tail vein. The dosage and calculations were according to MILLER and TAINTER (1944). Each type of emulsion was administered to at least 6 groups of 10 mice each. The dosage is shown in Fig. 2. The concentration of the emulsions was chosen so that the required quantities of triglyceride could be administered in volumes between 2 and 40 ml/kg. Thus triacetin, tricaprylin, trinonylin, tricaprinin and triundecylin were investigated as 25 % emulsions, while the remainder were prepared as 10 per cent emulsions. Before injection the triisovalerin and tricapronin emulsions were diluted to a final concentration of 2.5 % and triheptylin to 5 % by the addition of appropriate volumes of 5 % glucose.

The results of the various $LD_{50}$ determinations are shown in Table I.

## Results.

It is apparent from Fig. 1 and Table 1 that there was a great difference in the toxicity of the various triglycerides. The lowest $LD_{50}$, 82 ± 4 mg/kg, was noted for triisovalerin. The other extreme was represented by trinonylin, tricaprinin and triundecylin, with $LD_{50}$ over 10 g/kg, *i. e.*, so high that it could not be determined.

$LD_{50}$, which showed a distinct minimum in triisovalerin, thus increased when the length of the fatty acid chain was less or more than 5.

Injection of triacetin, tripropionin, tributyrin and triisovalerin produced almost immediate convulsions, failure of the righting

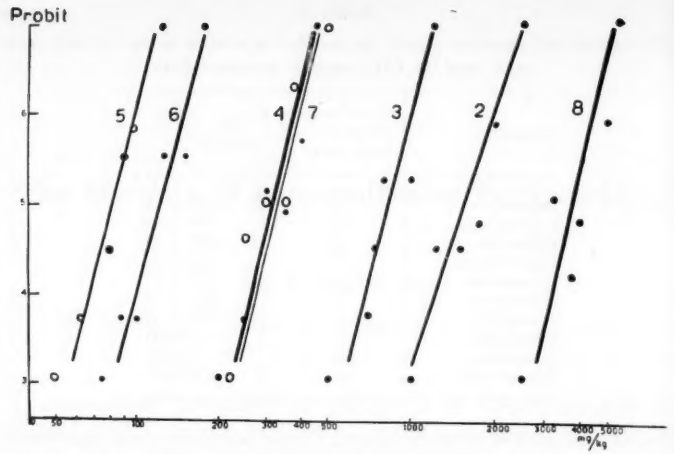


Fig. 1. *Dose-mortality diagram of low-molecular triglycerides on intravenous injection in mice.* The probit value is given on the ordinate, and the number of mg/kg of body weight of the different triglycerides on the abscissa. Each dot represents a group of 10 mice. The figures denote the number of carbon atoms in the fatty acid part of the different triglycerides, as listed in Table I. The lines for 4 and 7 coincide, ● denoting glyceride 4, and ○ glyceride 7.

reflexes and respiratory arrest. In some animals respiration did not return and death occurred in 1—3 minutes; other animals started to breathe spontaneously within a few minutes and survived.

When tricapronin was injected intravenously, clonic and tonic spasms appeared at once, particularly in the hind legs; there was respiratory arrest and loss of righting reflexes. In some cases they died after a few minutes. In other cases the symptoms disappeared rapidly and it seemed as if the animals would survive. If, however, the dose was close to $LD_{50}$ five or ten minutes later spasms and respiratory embarrassment would reappear, the animals would again lose the righting reflexes, show frothing from the nose and usually die.

Triheptylin gave rise to a different symptom complex. Directly after the injection, motor uneasiness developed. This was followed by spasms in the hind legs, respiratory embarrassment, urination and assumption of the lateral position, as well as frothing at the nose. Higher doses generally led to immediate death, but with doses around $LD_{50}$ many animals survived these initial symptoms,

although they died 1—5 hours later, and frothing at the nose was then observed.

The symptoms observed following the injection of tricaprylin were largely the same as those produced by triheptylin.

When trinonylin was given in a dose of 10,000 mg/kg, increased respiration and some restlessness appeared, followed by lethargy. With the same dose of tricaprinin and triundecylin, no effect could be observed.

## Discussion.

The present investigation has shown that certain pure neutral fats exhibit an appreciable toxic effect when administered intravenously in the form of an emulsion. The remarkable feature is the great difference in the toxicity of the several triglycerides. The $LD_{50}$ has a distinct minimum for the glycerides containing fatty acids with 4, 5 or 6 carbon atoms and increases abruptly as the number of carbon atoms increases or decreases. Great changes in the pharmacological effectiveness of various *water-soluble* substances *e. g.* methonium compounds are known result when the number of carbon atoms is either increased or decreased in an otherwise analogous series. In triglycerides, which are completely insoluble in water, these great differences in biologic activity are unexpected. A possible explanation is that the injected fat particles because of difference in their charge are differentially attracted to certain cells or that once these fatty substances are incorporated into the lipophilic layer of the cell membrane they might alter the cell potential with resulting pharmacological effects.

Further detailed investigations are necessary before the lethal effect of the different glycerides can be explained pharmacologically. Some aspects of this matter have been studied in the case of tributyrin (Wretlind 1957). It was found that death of the experimental animals (mice) could probably be attributed to apnoea, elicited reflexly from receptors in the right side of the heart, or in the region supplied by the pulmonary artery, their fibres running centrally through the vagus.

The high toxicity of certain low-molecular weight glycerides found in the present study is in agreement with the results which Shafiroff, Mulholland and Baron (1952) reported on the basis of studies with parenteral emulsions in man. They split

coconut oil into free fractions. Fraction A, which contained glycerides of low-molecular fatty acids such as capronic acid, produced severe toxic effects in the form of dyspnoea, cough with expectoration of foamy sputum, vomiting, etc., when injected intravenously as a 10 per cent emulsion. Fractions B and C, containing glycerides of fatty acids with a larger number of carbon atoms, could be administered intravenously without noticeable side effects.

Only fatty acids with an even number of carbon atoms are found in nature. Consequently, some biologic differences might be expected between neutral fats containing fatty acids with an even and an uneven number of carbon atoms, respectively. In nutritional experiments in rats, OZAKI (1926) found a considerable difference between various glycerides containing low-molecular fatty acids. In general, glycerides with fatty acids containing an uneven number of carbon atoms had a lower nutritional value than the natural fats. Furthermore, he found that tripropionin and triisovalerin exhibited marked toxicity with a lethal effect when they constituted 5 per cent of the diet. SNYDERMAN, MORALES and HOLT Jr. (1954) made nutritional tests in premature infants, and showed that better growth was produced with a synthetic diet containing 34 per cent tributyrin or triacetin than with one containing butter-fat. No toxic effects were observed.

It is thus apparent from both these investigations that certain low-molecular glycerides of fatty acids with an even number of carbon atoms are suitable for nutritional purposes when given orally. This is in contrast to the results obtained with intravenous administration, where the low-molecular glycerides exhibited considerably higher toxicity. Moreover, no systematic differences were observed between the toxicity of glycerides with fatty acids containing an even and an uneven number of carbon atoms. The probable explanation of this discrepancy is that in oral administration it is a question of an effect on the fat metabolism, the organism having difficulty in dealing with so-called unnatural fatty acids. In the acute toxicity associated with intravenous administration, it is a question of pharmacological effects that are elicited directly, without the occurrence of any catabolic or anabolic processes.

The toxicity of the low-molecular glycerides is evidently dependent on the mode of administration. It can be inferred from the aforegoing that entirely different results are obtained when

they are administered orally in the diet, and when they are injected intravenously. LI, SAH and ANDERSSON (1941) determined the $LD_{50}$ of triacetin on subcutaneous injection in mice; it was found to be 2.3 ml/kg, *i. e.*, about 1.5 times as great as the intravenous $LD_{50}$ dose.

## Summary.

The $LD_{50}$ of low-molecular triglycerides has been determined by intravenous injection into mice. The triglycerides were administered in the form of emulsions.

The $LD_{50}$ is found to be lowest in triisovalerin ($82 \pm 4$ mg/kg) and to increase when the fatty acid chain contains less than 5 carbon atoms, reaching $1{,}600 \pm 81$ mg/kg in triacetin. With more than 5 carbon atoms in the fatty acid chain, $LD_{50}$ rises successively and lies over 10,000 mg/kg for trinonylin, tricaprinin and triundecylin.

With lethal doses the symptom complex is somewhat variable, but generally includes respiratory arrest or respiratory embarrassment with a rapid onset, this being the probable cause of death.

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# The Mechanism of the Vasodilator Effect of Adrenaline.

## II. Influence of Anesthetics on the Depressor, Pressor and Lactic Acid Stimulating Effects of Adrenaline.

By

LENNART LUNDHOLM.

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In the preceding investigation in this series (LUNDHOLM 1957) the vasodilator effect of adrenaline in skeletal muscle was shown to be associated with, and dependent on, its stimulatory effect on the lactic acid production therein. This vasodilatation in skeletal muscle was accompanied, at least in anesthetized animals, by a depressor effect, but the latter's magnitude, like the vasodilatation in skeletal muscles, was largely dependent on the anesthetic employed. The depressor effect was greatest in animals anesthetized with ether, chloroform and urethane; weaker in those with chloralose (MACDONALD and SCHLAPP 1926, VINCENT and CURTIS 1927), and uncertain in animals anesthetized with barbiturates, or decerebrated or unanesthetized (BERRY 1917, GRUBER 1929, DRAGSTEDT 1928, HERWICK, LINEGAR and KOPPANYI 1938). Hence it was worthwhile investigating the influence of various anesthetics on the hypotensor and lactic acid producing effects of adrenaline, and comparing the results with the corresponding effects in unanesthetized animals. The lactic acid producing effect of noradrenaline too was studied with reference to the fact that it has no hypotensive effect in cats.

The investigations of CANNON and LYMAN (1913) had already shown the significance of the basal blood-pressure level for the magnitude of the depressor effect, and hence that factor was taken into account too.

Lastly, since the action of adrenaline on the blood pressure was the sum of its vasodilator and vasoconstrictor — cardiostimulatory — effects, variations in the magnitude of either of those components should be reflected in the total action; accordingly, even the pressor effect of adrenaline was correlated with its lactic acid stimulating effect under various experimental conditions. The basal blood-pressure level was also found to have a bearing on the magnitude of this pressor effect.

## Methods.

The experiments were performed on about fifty full-grown cats with body weights of between 2.5 and 5.5 kg and having no symptoms of infection. In small animals the depressor effect was usually feeble and the blood loss at withdrawal of samples caused a fall of blood pressure. When symptoms of infection were present, the basal lactic acid content was usually elevated.

The cats received initial ether anesthesia except in four of the dial experiments where that anesthetic was given initially. In the experiments with dial anesthesia alone and those in which it was combined with initial ether anesthesia, the basal lactic acid content showed no difference. The depth of anesthesia was as superficial as possible without symptoms of excitation. Since the signs of anesthesia varied considerably with the different anesthetics, the behavior of the reflexes will not be described in detail; the conjunctival reflex persisted in most animals and the corneal reflex, as well as the reflex ear twitch, in all cases. Various pain reflexes were elicitable too. In particular the anesthesia was superficial in the experiments with evipan, since these cats generally reacted even to acoustic stimuli. Broadly speaking, the depth corresponded to the first plane of surgical anesthesia. In those cases where the anesthesia was controllable — ether and evipan — it was deepened somewhat during preparation of the animals.

In the first few experiments ether anesthesia was produced by a previously described method (LUNDHOLM 1957). In the subsequent ones the animals breathed via a small Krogh spirometer filled with $O_2$ in which ether was dripped until the requisite depth of anesthesia was reached. This latter mode of administration was superior in that the depth of anesthesia could readily be kept constant, and in view of the adequate supply of $O_2$ the basal lactic acid content was low. The oxygen consumption, too, was measured with the aforementioned spirometer. In most of the experiments with other anesthetics than ether the cats inspired $O_2$ from this spirometer, since it tended to keep the basal lactic acid content low.

Other anesthetics were administered intravenously in the following doses: Chloralose in 1 per cent solution, 60 mg/kg; urethane in 20 per cent solution, 0.8—1.0 g/kg; dial as sodium salt in 10 per cent solution, 70 mg/kg. When dial was administered without preceding ether anesthe-

sia, it was by subcutaneous injection. Evipan was given as sodium salt in 2 per cent solution, with repeated injections as required for maintaining a constant depth of anesthesia.

The animals were then prepared as follows. A cannula was inserted into the trachea and a T cannula into the left carotid artery. One branch of the latter cannula was connected to a mercury manometer for blood-pressure recording. From the other branch — after occlusion of the branch to the mercury manometer and aspiration of 1 ml blood — samples of 1.5 ml were taken for lactic acid assay. For the intravenous infusions a plastic catheter was introduced into the right jugular vein and advanced to its junction with the subclavian vein. The blood flow in the skeletal muscles, when simultaneously determined, was measured by a previously described method (LUNDHOLM 1957).

In the experiments on unanesthetized animals the procedure differed somewhat. Under ether and local anesthesia with lidocaine, a plastic catheter was introduced into a branch of the right jugular vein, after which anesthesia was continued with evipan. Into another branch of the jugular a further plastic catheter was advanced into the right ventricle under fluoroscopic control. Into the right external carotid was inserted yet another plastic catheter, the tip of which lay a centimeter or so above the junction with the common carotid. The incisions were sutured and the catheters attached by adhesive tape round the neck. The cat was then placed in the respiration chamber of a metabolic apparatus which has been described earlier (LUNDHOLM 1949). This apparatus enabled the oxygen consumption to be determined, during which procedure the animals were remarkably placid in the confined space of the chamber. When the effects of anesthesia seemed to have worn off after 60—90 minutes, the experiments began. This method enabled not only the blood pressure but also the cardiac output to be determined by Fick's method.

After the completion of preparation, each animal received 1 ml of a 5 per cent heparin solution. In experiments with durations exceeding 2 hours, a further 0.5 ml heparin solution was administered after that time.

The infusions were given intravenously by means of an apparatus that injected a volume of solution which could be increased stepwise in geometric series; namely, 0.1, 0.3, 0.9 and 2.7 ml per min. The stock solution of adrenaline and noradrenaline was diluted according to body weight, so that the dose, too, increased in geometric series as follows: 0.1, 0.3, 0.9, 2.7, 8.1 and 24.3 $\mu$g per kg body weight per minute. The diluent had the following composition: 0.9 per cent NaCl, 0.05 per cent $Na_2S_2O_5$, and 0.1 N HCl to pH 4.0. Intravenous infusion of up to 5 ml per minute of this solution did not influence the blood pressure or muscle blood flow.

Synthetic l-adrenaline (BIOS) and l-noradrenaline (WINTHROP and STERN) were used for the experiments. The doses refer to the amount of free base.

The lactic acid content of arterial blood was determined by the method of FRIEDEMANN and GRAESER (1933) on 1.5 ml blood. In each case duplicate determinations were made.

The $O_2$ content of arterial and venous blood was determined according to VAN SLYKE and CULLEN (PETERS and VAN SLYKE 1932) on 0.5 ml blood.

## Results.

In the preliminary experiments adrenaline was injected rapidly in a dose of 2 $\mu$g in ether-anesthetized cats. The depressor effect that emerged (fig. 1 A) was attended by a rise of $0.76 \pm 0.51$ mg per cent (mean of nine experiments) in the lactic acid content of the blood. The rise therefore bordered on that which could be determined reliably by the analytical method. For this reason I changed to slower intravenous infusion of adrenaline, which procedure had earlier been found to produce a much greater increase of lactic acid (LUNDHOLM 1949). Continuous infusion of adrenaline proved superior to rapid injection in other respects too. While the depressor effect was initially fairly uniform under the different experimental conditions, its duration varied substantially. Moreover, the correlation of the depressor and pressor effects, as well as the lactic acid production, to the adrenaline concentration should be far more distinct with infusion than after rapid injection of adrenaline. With this latter a fairly high concentration of adrenaline arises in a certain volume of blood. The tissue contiguous to the blood stream — the smooth-muscle layer of the blood vessels — may therefore be exposed to a considerably higher concentration of adrenaline than are more distant tissues, as for instance the skeletal muscle cells. Hence the relation between the direct vasoconstrictor effect of adrenaline and the indirect dilator effect via lactic acid production in the skeletal muscle may be shifted in favor of the former.

In the quantitative experiments, adrenaline was therefore infused intravenously in doses of 0.1, 0.3, 0.9, 2.7, 8.1 and 24.3 $\mu$g/kg body weight/min for 5 minutes. Infusion always began with the lowest dose, after which the dose was gradually increased. An interval of exactly 5 minutes was allowed between the infusion periods. Immediately before the first infusion period, arterial blood was taken for lactic acid assay; then further samples were taken 2—3 minutes after each period. When the infusion had terminated, further blood samples were often taken at intervals of 10—20 minutes, for determination of the lactic acid elimination rate.

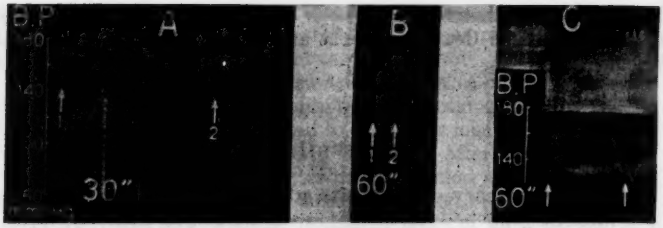


Fig. 1. A. Cat. 5.0 kg, under ether anesthesia. (1) 2 μg adrenaline intravenously; (2) 40 mg dl-lactic acid intravenously. Carotid arterial pressure.

B. Cat, 3.4 kg, under ether anesthesia. (1) Injection of 4 mg lactic acid into the aorta via a catheter inserted through the left carotid artery. (2) Injection of 4 mg lactic acid through a T cannula introduced into the right carotid. Blood pressure measured in the femoral artery.

C. Cat, 5.0 kg, under dial anesthesia 60 mg per kg. Upper curve shows the blood flow in the right foreleg, registered by drop recorder; lower curve, the carotid arterial pressure. Between the arrows, intravenous infusion of 0.9 μg adrenaline/kg/min.

To ensure duplicability of adrenaline's effect on the lactic acid content of the blood, it was essential that the initial content was basal. The value that showed no change on repeated determinations was defined as the basal lactic acid content. Values exceeding 25 mg per cent invariably tended to fall with repeated determinations. For this reason, experiments in which the initial lactic acid value exceeded that figure were discarded. Only in the evipan experiments was it necessary to use higher basal values, since they were found to be constant and only in one of five cases were they below 30 mg per cent. BUNKER, BEECHER, BRIGGS, BREWSTER and BARNES (1951), in experiments on dogs, found that the lactic acid content regularly increased under ether anesthesia. In my cat experiments this appeared to be the case with deep ether anesthesia, but under lighter anesthesia ether did not appreciably increase the lactic acid content, especially if the animals inspired pure $O_2$.

For estimation of the blood pressure response the average fall and rise were determined during the infusion. The area enclosed by the blood-pressure curve and the base line, plotted with respect to the blood pressure before and after infusion, was determined planimetrically and the average change of blood pressure calculated. In those cases where an initial blood-pressure fall was succeeded by a secondary rise, the two areas were measured separately and the mean depressor or pressor effects computed for the whole of the 5-minute period.

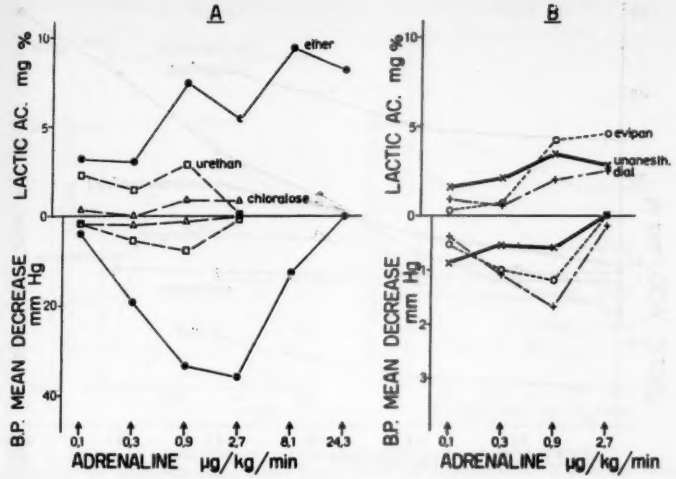


Fig. 2. Association between lactic acid increase in the blood and average relative blood-pressure fall (in relation to initial blood pressure) during infusion of adrenaline in increasing doses. Adrenaline was infused for 5-minute periods, with 5-minute intervals, in rising doses from 0.1 µg/kg/min to 24.3 µg/kg/min. Upper part of the figure shows average increase in lactic acid content of the arterial blood that emerged during each infusion period. In fig. A those experiments are collected in which the initial blood pressures were more or less alike and the depressor effect was chiefly dependent on the magnitude of the lactic acid production. In fig. B, not only the lactic acid production but also the initial blood pressure varied; since, moreover, the blood-pressure regulation was better in the unanesthetized animals, the correlation between lactic acid production and depressor effect was not so marked in these experiments. Note that the linear scale for the depressor effect is over ten times greater in B than in A.

In addition to these changes of blood pressure in relation to the basal level — hereinafter called relative blood-pressure changes — the absolute minimum or maximum, in millimeters of mercury, reached during the infusion was also recorded; in the following it is called the absolute blood-pressure fall or rise.

*Unanesthetized Animals.* Experiments were performed on five different cats with infusion of adrenaline in doses of 0.1—2.7 µg/kg/minute. At the lower doses a slight depressor effect emerged (fig. 2 B) which in maximal cases amounted to 4 mm Hg, though in two cases it was clearly connected with the infusion and hence did not merely reflect spontaneous variations in blood pressure. The threshold dose for the pressor effect was 0.3 µg/kg/min where a rise of blood pressure occurred in four of five cases (fig. 7). The initial lactic acid content averaged 19.7 mg

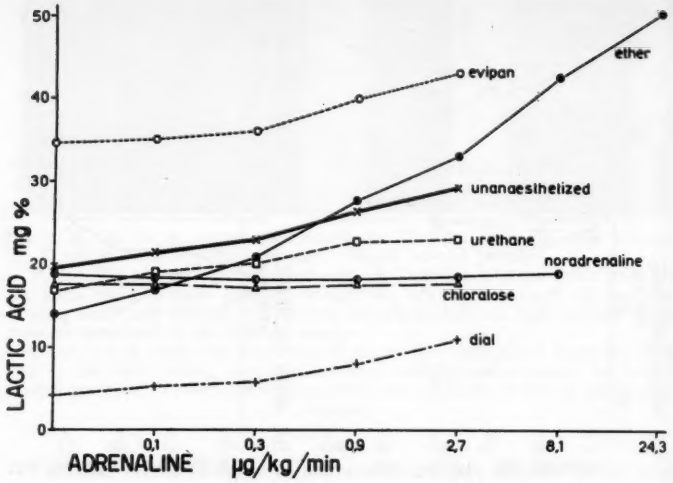


Fig. 3. Basal values for the lactic acid content of arterial blood and its changes during infusions of adrenaline and noradrenaline.

per cent, and it was distinctly raised even by 0.1 $\mu g$ adrenaline (fig. 3).

The depressor effect of adrenaline in the unanesthetized animals was very slight by comparison with the others, but it does not follow that adrenaline had no vasodilator action in the former group. In an experimental series of three unanesthetized cats where the effect of adrenaline 1.0 $\mu g$/kg/min, infused intravenously, was determined on the cardiac output, it was found (fig. 4) that while the latter increased by an average of 25 per cent, the blood pressure rose by a maximum of only 10 per cent, pointing to a decrease in the total peripheral resistance. In subsequent experiments on unanesthetized rabbits, adrenaline increased the cardiac output and reduced the peripheral resistance even in doses that failed to influence the blood pressure (Part III).

*Urethane Anesthesia.* Animals in which the effect of adrenaline had been studied while they were not anesthetized, were subjected, about 14 days later, to similar experiments under urethane anesthesia. The basal lactic acid content and the lactic acid stimulating effect of adrenaline were largely the same as they had been without anesthesia; only after the largest dose of adrenaline was the lactic acid production apparently weakened.

Fig 4

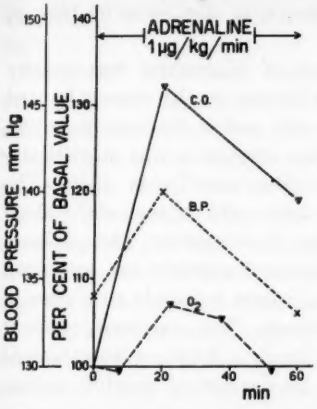


Fig 5

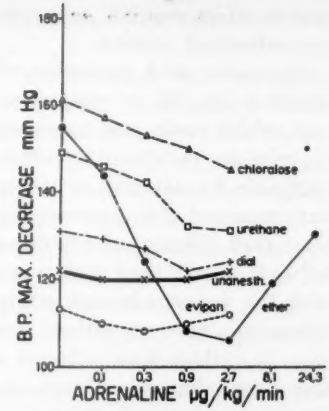


Fig. 4. Effect of adrenaline on cardiac output, blood pressure and oxygen consumption in unanesthetized cats. Mean of three experiments. Adrenaline was infused intravenously in a dose of 1.0 µg/kg/min for 60 minutes. Cardiac output was determined 5 minutes before infusion and, in two cases, after infusion for 25—30 minutes; and in all three experiments, after infusion for 55—60 minutes. The average basal cardiac output was 111 ml/kg/min (99, 106 and 128 ml/kg), and the basal oxygen consumption, determined for 30 minutes before the infusion, averaged 7.9 ml/kg.

Fig. 5. Relationship between dose of adrenaline and average maximal fall of blood pressure recorded at some time during the infusion. In those experiments where only a pressor effect occurred, the value for initial blood pressure immediately before infusion is shown.

The basal blood pressure, on the other hand, was increased by an average of 27 mm Hg; the depressor effect of adrenaline was greatly augmented and, in most cases, persisted throughout the infusion. In maximal cases the blood pressure fell by 28 mm Hg, compared with 4 mm Hg in the unanesthetized animals. The threshold dose for the pressor effect was 2.7 µg, *i. e.*, it had risen ninefold under the influence of urethane.

*Chloralose Anesthesia.* In the six experiments on animals anesthetized with chloralose the initial blood pressure was high, averaging 161 mm Hg. This accorded with the observation by BRENDEL, KOPPERMANN and THAUER (1954) that chloralose raised the blood pressure in dogs. — In two animals adrenaline had no depressor effect and in the others the blood-pressure fall, though initially quite distinct (maximum of 28 mm Hg), was of short duration. Only in one case did a depressor effect persist throughout the infusion. In the other cases the blood-pressure

fall was usually followed by a rise. The threshold dose for the pressor effect was 0.3 μg adrenaline, *i. e.*, the same as that in unanesthetized animals.

The lactic acid producing effect of adrenaline was greatly inhibited (fig. 32) by comparison with that in the unanesthetized cats, which result was consistent with earlier findings in rabbit experiments (LUNDHOLM 1949). This inhibition was statistically verifiable by analysis of variance (BONNIER-TEDIN 1940). The data consisted of the increase in the lactic acid content of the blood after each infusion of a given dose of adrenaline. The variance between anesthetized and unanesthetized animals was compared with the variance in each group of values referable to a dose of adrenaline with or without anesthesia. The variance quotient was 15.1; there was 1 degree of freedom between anesthetized and unanesthetized animals, and 34 degrees of freedom within the groups; hence $P < 0.001$.

*Dial Anesthesia.* The basal blood pressure in these experiments was virtually identical with that in the unanesthetized animals. The depressor effect of adrenaline emerged in four of the seven experiments but, as in those with chloralose, it was of short duration; only in one case (fig. 1 C) did it persist throughout the infusion period. The maximal fall of blood pressure amounted to 24 mm Hg. The pressor effect was distinctly augmented by comparison with unanesthetized animals, as was evident both from the threshold dose for a pressor effect (0.1 μg) and from the absolute blood-pressure rise. KOPPERMANN, BRENDEL and THAUER (1955) found, in dogs, an amplified pressor effect of adrenaline under pernocton anesthesia.

The basal lactic acid content of the blood was much lower (4.3 mg per cent) under dial than under other anesthetics. Yet the effect of adrenaline on the lactic acid content was considerably weaker than that in unanesthetized animals, this applying in particular to the lower doses. This inhibition was not statistically verifiable by analysis of variance as in the chloralose experiments, though it was verified if the lactic acid elimination rate was taken into account.

*Evipan Anesthesia.* The basal blood pressure was somewhat lower under evipan than in the unanesthetized cats, which finding accorded with the observations of BRENDEL, KOPPERMANN and THAUER (1954) in dogs. In two of the five experiments, no depressor effect was obtained. In the others the blood-pressure

fall was initially distinct (maximum 8 mm Hg) but of short duration. The pressor effect was like that in unanesthetized animals.

The basal lactic acid content was elevated by comparison with the experiments without anesthesia. Adrenaline's lactic acid producing effect was somewhat weaker in the lower doses, but stronger in the higher ones, than that in the unanesthetized animals; the total effect in this respect was therefore unchanged.

*Ether Anesthesia.* Here the initially high blood pressure — averaging 155 mm Hg — accorded with the observations of BREWSTER, ISAACS & WAINÖ-ANDERSON (1953) and others, that in dogs the blood pressure increased under superficial ether anesthesia. The blood-pressure response was dominated by the powerful depressor effect, which persisted throughout the infusion and reached 70 mm Hg in maximal cases. The depressor effect increased almost linearly with the logarithm of the dose up to 2.7 $\mu$g adrenaline/kg/min. At 8.1 $\mu$g the pressor effect emerged in one of the five experiments. After 24.3 $\mu$g there was an initial rise of blood pressure in all cases, though it was followed by a substantial and prolonged depressor effect after the end of infusion.

The basal lactic acid content was equal to that in the unanesthetized cats, but the lactic acid producing effect of adrenaline was considerably augmented. Analysis of variance as in the chloralose experiments gave a variance quotient of 4.4, with 1 degree of freedom between ether-anesthetized and unanesthetized animals, and 30 degrees of freedom within the groups; $P < 0.05$. Augmentation by ether anesthesia of the lactic acid producing effect of adrenaline was thus statistically probable.

In four experiments adrenaline was infused intravenously in a dose of 0.9 $\mu$g/kg/min for 60 minutes in ether-anesthetized cats, and the effect on the blood pressure, oxygen consumption, and lactic acid content of the blood was determined. The results are shown in fig. 6, which gives the mean values from the four experiments. During the first 5 minutes the blood pressure showed a comparatively substantial fall, which thereafter decreased, but persisted even after the infusion had terminated. Not until the lactic acid content of the blood had returned to the basal level was the blood pressure normalized. Apparently, therefore, some correlation existed between blood pressure and lactic acid content, though it was not always so distinct as in these experi-

ments: only at times was it possible by intravenous infusion of lactic acid to produce a blood-pressure fall in the ether-anesthetized cat. In unanesthetized rabbits, however, such infusion regularly produced vasodilatation (Part III).

The lactic acid content of the blood increased to a maximum value of about 30 mg per cent, concomitantly with a rise of 16 per cent in the oxygen consumption. The correlation earlier demonstrated in rabbits (LUNDHOLM 1949) between adrenaline's stimulating effect on the oxygen consumption and its lactic acid producing effect was thus evident in these experiments too. Cats, however, seemed to oxidize lactic acid faster than did rabbits; for in the latter animals the lactic acid content of the blood had to increase to 50—60 mg per cent for a rise of 15 per cent in the oxygen consumption.

*Noradrenaline.* In five ether-anesthetized cats I studied the effect of noradrenaline in a dose of 0.1—2.7 μg/min on the blood pressure and lactic acid production. In common with earlier investigators, I found that noradrenaline had no depressor effect in cats. It too had no stimulating effect on the lactic acid production (fig. 3). The threshold dose for the pressor effect was 0.1 μg/kg/min.

In this connection it is worth while pointing out that isopropylnoradrenaline stimulated the lactic acid production to the same degree as adrenaline (LUNDHOLM, to be published).

### *Elimination Rate of Lactic Acid in the Blood.*

The basal lactic acid content of the blood was a dynamic state of equilibrium between the production — localized chiefly to the skeletal muscles and erythrocytes — and the elimination, localized mainly to the liver, heart and lungs. With an increased lactic acid production, the content in the blood initially rose but, as shown in fig. 6, a new state of equilibrium gradually arose where the augmented production was compensated by increased elimination. The blood's content of lactic acid was therefore in some degree a pointer to the metabolic rate for lactic acid in the organism, and the rate of elimination was dependent on the content in the blood (LUNDHOLM 1949). It is evident from fig. 3, however, that the basal lactic acid content varied under the different anesthetics, and so did the level reached during infusion. The elimination rate, therefore, could not be assumed to have

**Table 1.**

*Rate of elimination of lactic acid as a function of lactic acid level of the blood.*

| Lactic acid content of the blood, mg% | 10—19 | 20—29 | 30—39 |
|---|---|---|---|
| No. of determinations ..... | 5 | 17 | 9 |
| Elimination, mg% per min. ..... | 0.12 | 0.30 | 0.41 |
| Standard error of the mean ..... | ± 0.021 | ± 0.036 | ± 0.073 |

been identical in all experiments, and hence the curves in fig. 3 were not directly comparable. Further, the anesthetics conceivably influenced the blood's content of lactic acid by directly inhibiting its elimination. Accordingly, I determined the rate at which the lactic acid content fell after termination of the adrenaline infusion. Such determinations were made under each of the relevant types of anesthesia, though most of them under ether, urethane and dial. The elimination rate varied from case to case, but no discrepancies referable to the different anesthetics were demonstrable. On the other hand, the elimination rate was evidently dependent on the lactic acid content of the blood (table I), which finding accorded with my earlier results in rabbits (LUNDHOLM, 1949). With the aid of these values for the elimination rate, it was possible to calculate the level to which the blood's content of lactic acid would have risen if the elimination could have been disregarded. When this increase was calculated for adrenaline doses of 0.1—2.7 $\mu$g/kg/min, and assuming the elimination to have proceeded for 30 minutes at rates set forth in table I, the following total increase in lactic acid content was obtained: $18.3 \pm 1.73$ mg per cent for unanesthetized animals; $15.0 \pm 2.27$ mg per cent for urethane; $20.3 \pm 3.42$ mg per cent for evipan; $9.9 \pm 1.45$ mg per cent for dial; $4.1 \pm 0.71$ mg per cent for chloralose; and $28.1 \pm 5.8$ mg per cent for ether. In relation to the previous statistical analysis the difference ($8.4 \pm 2.26$) between unanesthetized and dial-anesthetized animals was statistically verifiable ($P < 0.01$) as was inhibition of the lactic acid production by chloralose. No definite effect of urethane or evipan was demonstrable, nor was the potentiating effect of ether fully verifiable statistically.

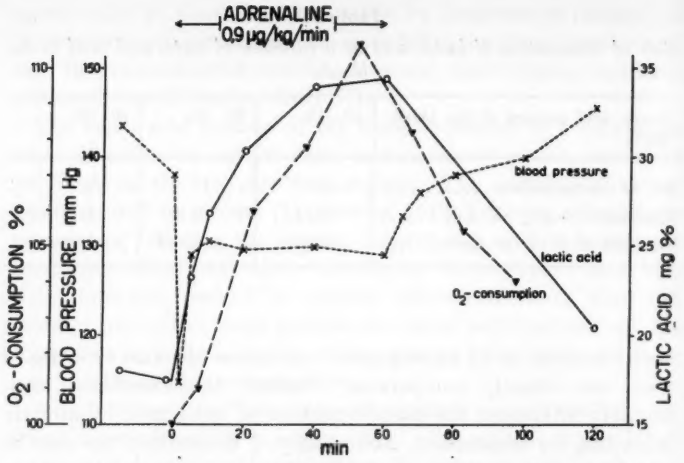


Fig. 6. Effect of adrenaline on blood pressure, lactic acid content of the blood, and oxygen consumption in ether-anesthetized cats. Adrenaline was infused intravenously in a dose of 0.9 µg/kg/min for 60 minutes. Mean values from four experiments. Basal oxygen consumption was determined for 30 minutes before the infusion and averaged 8.1 ml/kg/min.

*Lactic Acid.* In the experiments on skeletal muscle vessels it was possible, by intra-arterial infusion of lactic acid, exactly to duplicate the vasodilator effect of adrenaline (LUNDHOLM 1957). In the experiments on whole animals, this was practicable only to a limited degree. The depressor effect produced by rapid injection of adrenaline was fully duplicable by an intravenous or intra-arterial (aorta) injection of 10 mg lactic acid per kg body weight (fig. 1 A). This was equivalent to the amount of lactic acid that adrenaline liberated per minute in the skeletal muscle (LUNDHOLM 1957). The depressor effect was accompanied by vasodilatation in the skeletal muscles. It was not always possible, however, to elicit a depressor effect by injection of lactic acid, nor indeed, other than in exceptional cases, to secure by continuous intravenous or infusion of lactic acid in to the aorta a depressor effect lasting throughout the infusion. In general such an effect occurred only during the initial stage of the infusion. In those cases where lactic acid was administered intravenously, the transient effect was conceivably due to buffering of the lactic acid by the blood bicarbonate, the $CO_2$ produced being eliminated in the pulmonary circulation. This hypothesis

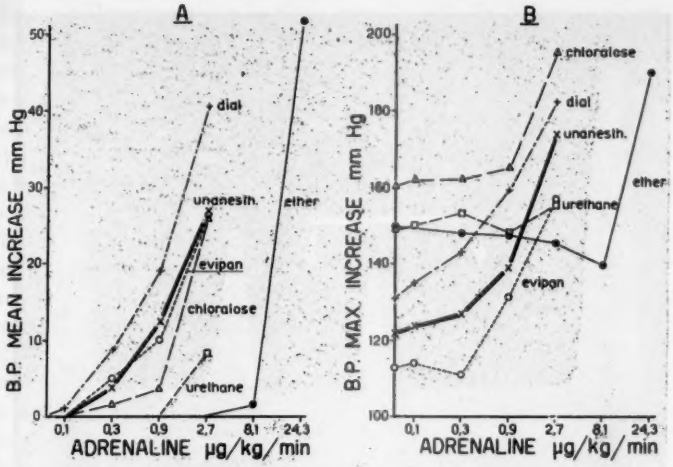


Fig. 7. Relationship between dose of adrenaline and pressor effect.
A. Average relative blood-pressure rise (in relation to initial pressure) during infusion of adrenaline. Note that the average pressor effect has been calculated for the entire 5-minute period even in cases where the blood-pressure rise lasted only for a part of it.
B. Average absolute rise of blood pressure. These values are based on the highest level reached by the blood pressure during infusion. Where no pressor effect occurred, the initial blood-pressure value immediately before infusion is taken.

was not valid, however, for intra-arterial administration of lactic acid; here the probable explanation of the brief depressor effect was that the peripheral vasodilator action of lactic acid was counteracted by its stimulation of the vasomotor center.

Figure 1 B shows that the depressor effect of 4 mg lactic acid, administered through a catheter introduced into the thoracic aorta via the left carotid artery, was almost identical with the pressor effect of a similar amount injected into the right carotid through a ⊤ cannula. However, the lactic acid infused into the aorta also reached the spinal sympathetic centers, which, like the center in the medulla oblongata, are stimulated by that substance (MATHISON 1910); this should explain why even infusion into the aorta had only a transient depressor effect.

It is unlikely, in any case, that the lactic acid production induced by adrenaline could be exactly duplicated by infusion of lactic acid. Infusion of adrenaline, as shown earlier (LUNDHOLM, 1957, page 43), caused during the first 10 minutes a relatively slight increase of the lactic acid elimination in the blood (lactic

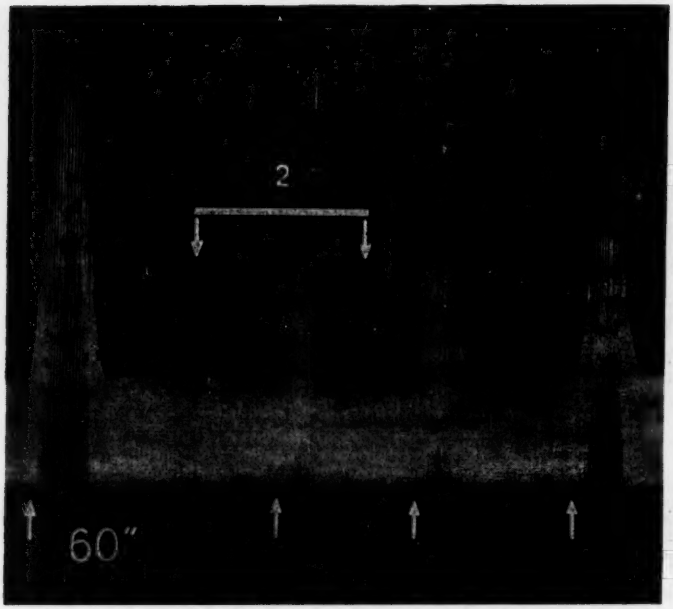


Fig. 8. Isolated rabbit ear perfused with Krebs solution. Flow rate determined with drop recorder. At each arrow, injection of 0.1 $\mu$g adrenaline. At 2, perfusion with Krebs solution containing 150 mg per cent ether.

acid was retained in the skeletal muscles); instead, the $CO_2$ elimination rose substantially. Since $CO_2$ was eliminated with passage of the blood through the pulmonary circuit, the increased $CO_2$ and lactic acid production induced by adrenaline infusion in the skeletal muscles had a purely peripheral vasodilator effect without stimulation of nervous centers.

*Vasodilatation and Depressor Effect.* In some experiments the relationship was studied between the vasodilatation in skeletal muscles on intravenous infusion of adrenaline, and the depressor effect. The relationship was quite evident in some cases, and the blood pressure curve reflected in detail the changes of blood flow in the skeletal muscles (fig. 1 C). In a few experiments on ether-anesthetized cats the depressor effect with low doses of adrenaline was nevertheless associated with a decreased blood flow in the foreleg muscles, and not until the dose was increased

did vasodilatation occur in those muscles too. In all cases where the blood flow was measured in hind-leg muscles, the depressor effect and increase of blood flow were parallel; hence there is reason to assume that the threshold dose for the vasodilator and lactic acid producing effects of adrenaline may vary in different types of skeletal muscle. In a few experiments on evipan-anesthetized cats, an adrenaline infusion in doses having no influence on the blood pressure was associated with an elevation of blood flow in the skeletal muscles. Adrenaline in doses of 0.3—2.7 μg/kg/min, which had a pressor effect, generally produced either an increase or no change in the blood flow; not until the doses reached 8—24 μg/kg/min did the blood flow decrease in the skeletal muscle. In those cases where the muscle blood flow increased at an elevated or virtually unchanged mean blood pressure, it may have been due not only to stimulation of the lactic acid production and increase of the perfusion pressure, but also to the reflex vasodilatation that adrenaline in high doses has been found to produce (GRUHZIT, FREYBURGER and MOE 1954, DÖRNER 1956). Summarizing, a depressor effect was regularly associated with vasodilatation in the skeletal muscles, but changes of vascular tone therein were not necessarily attended by alterations of blood pressure.

*Effect of Anesthetics on the Vasoconstrictor Action of Adrenaline.*

The magnitude of adrenaline's depressor effect should be influenced, too, by variations in the susceptibility of the muscle coat of blood vessels to its vasoconstrictor effect. LOCKETT (1951) reported that pentobarbital anesthesia weakened the pressor effect of adrenaline; BREWSTER, ISAACS & WAINÖ (1953) assumed that its vasoconstrictor action was weakened under ether anesthesia. In order to study the influence of different anesthetics on its vasoconstrictor effect, experiments were performed on isolated rabbit ear by perfusion with Krebs' solution that had been aerated with 5 per cent $CO_2$ in $O_2$. The experiments were conducted at room temperature. To the Krebs' solution were added the various anesthetics in the concentrations they were estimated to have had in the experimental cats' blood; *i. e.*, ether 150 mg per cent, urethane 100 mg per cent, dial 7 mg per cent, and chloralose 6 mg per cent. The perfusion rate was measured with a drop recorder according to CLEMENTZ and RYBERG (1949). The anes-

thetics reduced the basal vascular tone somewhat, and distinctly inhibited the vasoconstrictor effect of adrenaline (fig. 3). No significant difference was found in the degree of this inhibition after the different anesthetics; only the time required for the full effect to return varied, and was shortest after ether and longest after chloralose. These experiments therefore helped to explain why the depressor effect was stronger in anesthetized than in unanesthetized animals, but not why it varied under different forms of anesthesia.

## Discussion.

*Depressor Effect.* For statistical analysis of the factors influencing the depressor effect of adrenaline, that effect was correlated with the increase in the blood lactic acid, the dose of adrenaline and the initial blood pressure, after which the partial coefficients of correlation were calculated according to YULE and KENDALL (1947). This was necessary because, although the lactic acid increase and depressor effect showed a statistically significant correlation on simple analysis, both of them were also correlated to the dose of adrenaline. The analysis comprised all experiments ($n = 128$) on anesthetized and unanesthetized animals in the dose range of 0.1—2.7 $\mu$g adrenaline/kg/minute. The partial coefficient of correlation between depressor effect and lactic acid increase in the blood after elimination of any joint correlation to the dose of adrenaline and initial blood pressure was 0.45 and statistically significant ($P < 0.001$). That between depressor effect and initial blood pressure — after elimination of the lactic acid increase and dose of adrenaline — was also statistically significant ($r = 0.35$; $P < 0.001$). This confirms the conclusion reached by CANNON and LYMAN (1913) regarding the significance of initial blood pressure for the magnitude of the depressor effect.

The correlation between the blood-pressure fall and the rise in lactic acid after adrenaline must not be taken to imply that the depressor effect was initially correlated to adrenaline's stimulation of the lactic acid production in skeletal muscle; for in experiments on skeletal muscle the vasodilator effect was found to be directly dependent, not on the lactic acid production but rather on the rate at which the lactic acid content increased in that muscle. Under basal conditions there was equilibrium

between the amount of lactic acid produced in the muscle and that diffusing into the blood, since the diffusion gradient between muscle and lactic acid adapted itself to the production. If the latter was stimulated, this state of equilibrium was disturbed and the lactic acid content increased in the muscle until the diffusion gradient had increased in the same proportion as the lactic acid production. During the initial phase of adrenaline infusion, some proportionality could therefore be expected between the degree of lactic acid stimulation and the rate at which the lactic acid content rose in the muscle (LUNDHOLM 1957).

In these experiments it was possible, by infusing adrenaline for short times at certain intervals, to raise the lactic acid content of the muscle during infusion; and in the intervals the content returned to or approached its original level. In this way some correlation was maintained between the rate at which the muscle content of lactic acid altered and the degree of lactic acid stimulation. However, the adrenaline infusions influenced that content in yet another way. Because the lactic acid continuously rose in the arterial blood, the diffusion gradient between muscle and blood tended to fall. Compensating this, however, was the fact that the content increased as much in the muscle as in the blood. This factor amplified the correlation between alterations of lactic acid content in the blood and in the muscle. It was experimentally possible to demonstrate that an intravenous infusion of adrenaline influenced the lactic acid content of the muscle — and hence produced vasodilatation — in two different ways. From fig. 6 it will be seen that continuous infusion tended gradually to raise the blood lactic acid to a constant level. A similar result ought to be possible with discontinuous infusion of adrenaline, whereby the increase of lactic acid in the muscle due to a rise of that in the blood should be eliminated. It was found that repeated infusions of adrenaline in the same dose for 5-minute periods with 5-minute intervals had a gradually declining depressor effect; concurrently the increase in blood lactic acid was reduced. In particular the duration of the depressor effect decreased.

Even though lactic acid production and initial blood pressure should have been, broadly speaking, the factors of greatest importance for the magnitude of adrenaline's depressor effect, other contributory factors were nevertheless present.

Of special interest were the causes of the much weaker depressor effect in unanesthetized than in anesthetized animals.

The slight depressor effect that has been observed in unanesthetized individuals was not due to a weak vasodilator effect of adrenaline, since in doses that failed to influence the mean blood pressure, it reduced the total peripheral resistance substantially (BARCROFT and SWAN 1953, LUNDHOLM, to be published). The difference in depressor effect should rather be attributed to a better blood-pressure regulation in the unanesthetized individuals. The inhibitory effects that various anesthetics have been found to exert on the blood-pressure regulating reflexes elicited from baroceptors in the carotid and aortic bodies (for review, *vide* KOPPERMANN, BRENDEL and THAUER 1955, BROWN and HILTON 1956) may have some significance in this connection. Yet according to the above-mentioned authors, chloralose does not inhibit these reflexes; hence I sought to establish, in chloralose-anesthetized cats, whether the depressor effect was influenced by denervation of the baroceptors in the aortic and carotid bodies. No amplification or prolongation of that effect was demonstrable after denervation. Since the initial blood pressure was considerably elevated under chloralose anesthesia, it might be objected, however, that with an already increased sympathetic tone it would be more difficult for the organism to compensate for vasodilatation. The inhibitory influence of different anesthetics on adrenaline's vasoconstrictor action might also have had a bearing on the more distinct depressor effect in the anesthetized individuals. Summarizing, the reason for the more evident depressor effect in anesthetized than in unanesthetized animals probably lay in a poorer blood-pressure regulation in the former. To what degree this was due to inhibition of central or peripheral parts of the blood-pressure controlling reflex mechanisms could not be determined, and probably varied with different anesthetics.

*Pressor Effect.* The magnitude not only of adrenaline's depressor effect, but also of its pressor action, may well be dependent on the initial blood pressure and the lactic acid production, although the correlation in the latter instance should be negative. The partial coefficient of correlation between pressor effect and initial blood pressure, after elimination of any action of the adrenaline dose and lactic acid production, was — 0.32 and statistically significant ($P < 0.001$).

The correlation was a natural consequence of the approximately linear increase of adrenaline's pressor effect with the logarithm of the dose (fig. 7). If the initial blood pressure was low — *i. e.*,

the sympathetic tone and the noradrenaline content at the sympathetic nerve terminals in the muscle of the cardiovascular system were low — intravenous infusion of a small amount of adrenaline caused a proportionally (logarithmic) greater increase in the total content of sympathetic amines than it did if the blood pressure and sympathetic tone were high. On the other hand, the total content of sympathetic amines in the cardiovascular muscle was naturally greater in the latter instance. It followed that the relative blood-pressure rise — in relation to the initial pressure — should have been greater at a low than at a high sympathetic tone, but the absolute height of the blood pressure greater at a higher sympathetic tone. The interaction of these factors was most evident on comparison of the pressor effects under dial and under chloralose anesthesia (fig. 7). The relative pressor effect was greater under dial anesthesia, where the initial blood pressure was lower than that under chloralose, whereas the absolute level reached by the blood pressure was higher under the latter anesthesia.

The partial coefficient of correlation ($r = -0.1$) between lactic acid production and pressor effect, after elimination of any relation between the dose of adrenaline and the initial blood pressure, was not statistically verifiable. This was somewhat surprising because, for instance, inhibition of adrenaline's lactic acid producing effect by $Cu^{++}$ transformed its vasodilator action on skeletal muscle into a vasoconstrictor action (LUNDHOLM 1957). The very slight correlation between lactic acid production and pressor effect may have the following explanation. The resulting action ($S$) of adrenaline on the blood pressure was composed of (1) its stimulatory effect on the heart (C); (2) its effect on skeletal muscle vessels, where both the lactic acid producing and vasodilator effects ($D_m$) and the vasoconstrictor effect ($P_m$) might be manifested; and (3) the action on most of the other vascular areas of the body where only the vasoconstrictor effect ($P_b$) emerged. The total action may thus be expressed as $S = C + P_b + (P_m - D_m)$. In cases where the lactic acid production was correlated to the pressor effect, however, those experiments were selected in which $C + P_b + P_m > D_m$, *i. e.*, the lactic acid producing effect was of minor importance relative to other factors, so that it was scarcely surprising to find only a slight correlation. If, instead, the lactic acid production was correlated with the average blood-pressure response during

infusion of adrenaline (a depressor effect was here denoted by a minus sign, and in the case of a biphasic action the depressor was subtracted from the pressor effect), the correlation was statistically significant. The partial coefficient of correlation, after elimination of the correlation between adrenaline doses and initial blood pressures, amounted to $-0.31$, and $P < 0.001$.

The factors which, in this investigation, proved to have a bearing on adrenaline's pressor effect are distinctly reflected in fig. 7 B. Infusion of 2.7 $\mu$g adrenaline seemed to produce, in the vascular smooth muscle, a concentration of sympathetic amines in which the amount of nordarenaline liberated from sympathetic nerve terminals played a minor role. Under chloralose and dial anesthesia when the lactic acid production was inhibited, the blood pressure reached higher levels than those in the unanesthetized animals. Under evipan and urethane, with a largely unchanged lactic acid production, the blood-pressure rise was somewhat less than that in the unanesthetized animals; this probably because of the inhibitory effect on adrenaline's vasoconstrictor action. Under ether anesthesia, moreover, the lactic acid production was so augmented that no rise of blood pressure occurred at all.

Anesthetics doubtless influence the pressor effect of adrenaline in several other ways than via the initial blood pressure, lactic acid production, and by weakening the vasoconstrictor effect. It appears likely, therefore, that the ability of the organism to compensate for adrenaline's pressor action via different depressor reflexes varies under different anesthetics. Several barbiturates, though not dial, have a direct blocking effect on the vagus (Gruber, Gruber and Colosi 1938). Boniface, Brown and Kronen (1955) found that a number of different anesthetics reduced the contractility of the heart, which fact, too, should have a bearing on the pressor effect of adrenaline. It seems probable, however, that the effects of the anesthetics on the lactic acid production and initial blood pressure were, generally speaking, the chief factors influencing the magnitude of adrenaline's pressor effect.

This investigation showed too, however, that under no form of anesthesia was adrenaline's effect on the circulation identical with that found in unanesthetized animals, and that different anesthetics changed it within very wide limits. Furthermore, the relevant effect also varied with the depth of anesthesia produced by one and the same agent. Lastly, blood pressures that are unchanged or elevated to the same level under varying experi-

mental conditions, may conceal substantial differences in the cardiac output and distribution of the blood flow. Hence it is scarcely surprising that today, sixty years after the discovery of adrenaline, there is still no unanimity of opinion as to its action on the circulation. Only a general analysis of this action in the unanesthetized individual, similar to that conducted by BARCROFT and his associates (BARCROFT & SWAN, 1953) may be expected to yield unequivocal results and to enhance our knowledge of the circulatory effects under physiologic conditions.

## Summary.

The influence of different anesthetics on the depressor and pressor as well as the lactic acid stimulating effects of adrenaline were investigated in cats and compared with the corresponding effects in unanesthetized animals. Adrenaline was infused intravenously for 5 minutes in increasing doses ranging from 0.1 to 24.3 $\mu$g per kg body weight per minute.

In unanesthetized animals the depressor effect was very slight, although adrenaline reduced the total peripheral resistance; this probably because the blood-pressure regulation was better than in the anesthetized animals. In most of the latter the depressor effect emerged, but its magnitude varied considerably according to the anesthetic employed. Broadly speaking, the depressor effect showed a statistically significant correlation both to adrenaline's ability to increase the lactic acid content of the blood and to the basal blood-pressure level.

The same factors that amplified the depressor effect of adrenaline weakened its pressor effect. The latter was negatively correlated to the basal blood-pressure level and to the lactic acid producing capacity of adrenaline. Further, the anesthetic had a direct inhibitory effect on adrenaline's vasoconstrictor action.

In the individual cases the influence of the different anesthetics on adrenaline's circulatory effect was explained as follows. Ether amplified the lactic acid production and raised the basal blood pressure in relation to adrenaline's effect on unanesthetized animals, the depressor effect was very pronounced and the pressor effect greatly weakened. Urethane did not influence the lactic acid production, but elevated the basal blood pressure; the depressor effect was distinct but the pressor effect weakened. Evipan did not demonstrably influence either the lactic acid production or the basal blood pressure; the depressor effect was

somewhat greater and the pressor effect somewhat less than in unanesthetized animals. Dial inhibited the lactic acid production without definitely influencing the basal blood pressure; the depressor effect was weak but the pressor effect amplified. Chloralose inhibited the lactic acid producing action of adrenaline but concurrently raised the basal blood pressure; these effects on adrenaline's action tended to counterbalance each other.

Noradrenaline had neither a lactic acid stimulating nor a depressor effect on ether-anesthetized cats.

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# Food Preferences in Obesity Caused by Goldthioglucose.

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Obesity is known to be of multiple origin (BROBECK 1955, MAYER 1955). Nevertheless, whatever the cause in any one case might be, the development of obesity and its maintenance is caused by relative overeating. The quantitative aspects of food intake in obesity have been studied by several investigators (BROBECK 1946, MAYER 1953). There is, however, reason to believe that the qualitative content of the food is of great importance for the development and further course of the obesity (LUNDBAEK and STEVENSON 1947, STROMINGER, BROBECK and CORT 1953).

The present study deals with some aspects of the free selection of diets in mice made obese by a single injection of goldthioglucose.

## Methods.

Sixty-three control mice and 32 obese mice of both sexes of the ASw strain were used for the experiments. The animals were fed *ad libitum*. A standard diet (mouse crackers) was given, consisting of 66.5 % carbohydrate, 30 % protein and 3.5 % fat.

In the free selection studies three different diets were used, given at the same time to one and the same animal. The composition of each diet was roughly the same as used by MARSHALL and MAYER (1954) in other types of experiments. Thus, one diet contained a high caloric content of carbohydrate, one of protein and one on fat. The diet high in carbohydrate was composed of 64 % carbohydrate, 25 % protein

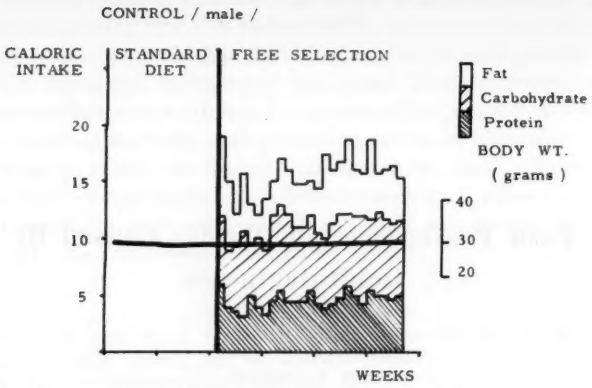


Fig. 1. Body weight on standard and free selection diets in a normal mouse.

and 11 % fat. The corresponding composition of the high protein diet was 69 % protein, 20 % carbohydrate and 11 % fat. Finally, the high fat diet contained 75 % fat and 25 % protein. In other words, whatever the food preference the intake of protein could never be less than 25 %. The three diets were given the mice in separate food jars. The food intake was measured at the same hour every day, and the body weights of the animals were, as a rule, checked every day. In some of the animals the water intake was measured daily.

In all the experiments the animals were housed in individual cages in a room with a temperature kept constant at 24° C by a thermostate.

Obesity was produced by a single injection of goldthioglucose (Solganal B-oleosum Schering). The amount injected to produce obesity was 1 mg per gram body weight. This dose was established by BRECHER and WAXLER (1949).

In one set of experiments the food preferences during pregestation, gestation and lactation were studied in control female mice of the same strain. Estrus was determined by vaginal smear examination or by observing the female's reaction towards the male.

Pathological studies have been made of sacrificed control and obese mice and in spontaneous deaths among the obese animals.

## Results.

As illustrated in Fig. 1 the change from the standard diet to free selection diets did not significantly influence the weights of the normal mice. As the different diets in the free selection experiments were composed, the intake of protein could never be

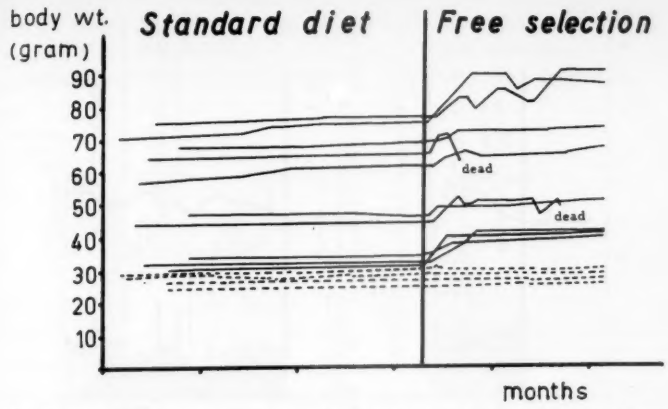


Fig. 2. Body weights of control mice (dotted lines) and obese mice (solid lines) on standard and free selection diets.

less than 25 %. The protein intake in the control animals averaged around 27 %. Of the remaining 73 %, carbohydrate and fat participated about equally to form the diet.

The food preferences in the obese animals varied so that no general rules could be set for the percentual consumption of the different nutrients. When the mice were on free selection, the body weight increased to a varying extent as shown in Fig. 2. The steadiness of the new higher weight plateau also varied considerably. Fig. 3 illustrates two different types of free selection in obese mice. One of the illustrated mice (31/55), when on free choice, gained about 15 grams, while eating almost exclusively fat — except for the obligatory 25 % protein. After the initial gain a steady weight was maintained and a preference for carbohydrate could be noticed. The other obese mouse (38/56) in this figure also gained about 15 grams — preferring fat — but after a weight plateau was reached, the preference for fat was still retained. This mouse was also an example of the lability of the weight and caloric intake. Another type of obesity is seen in Fig. 4 where no significant overweight was noticed on the standard diet but occurred on free selection. When returned to the standard diet, the external signs of obesity disappeared and reappeared on renewed free selection.

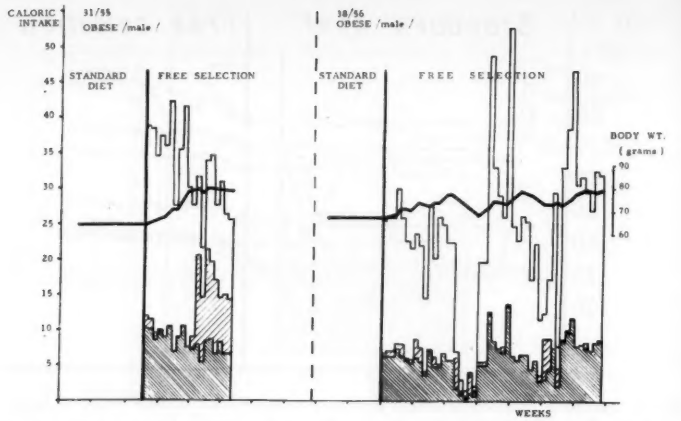


Fig. 3. Body weight on two obese mice on standard and free selection diets, illustrating the preferences for fat, carbohydrate and protein.

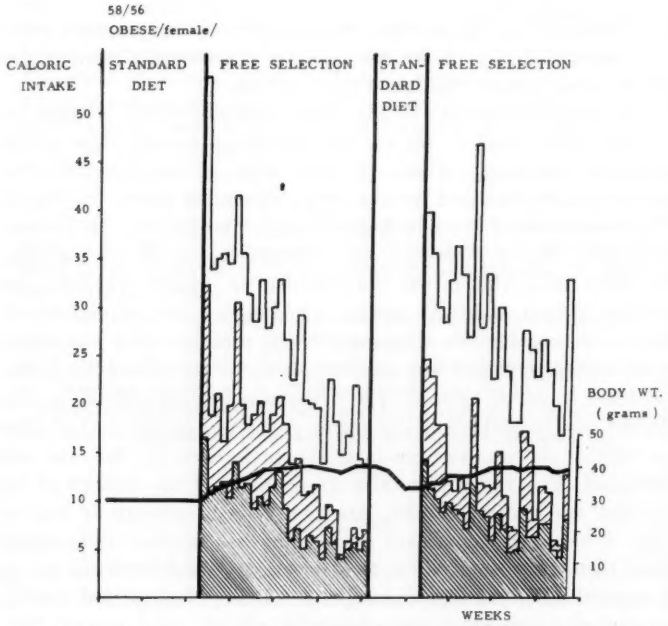


Fig. 4. Body weight and food preference in an obese mouse on alternating standard and free selection diets.

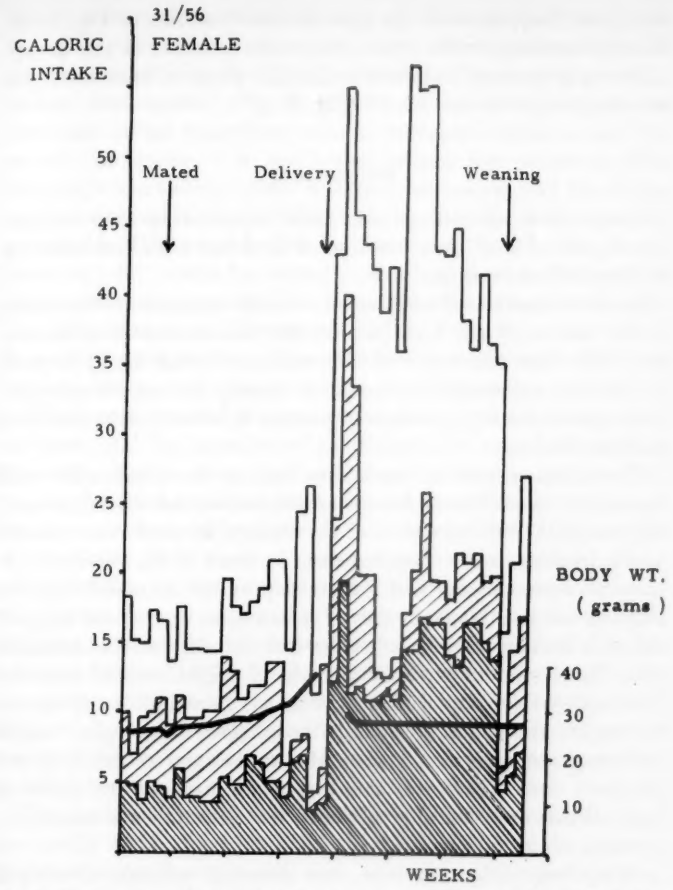


Fig. 5. Food preference of control female mouse on free selection diet during pregestation, gestation and lactation.

Among the obese animals the three main types of food preferences and weight characteristics noted above were about equally represented. When the intake of fat was highest steatorrhea was frequently observed. Four obese animals on the standard diet showed glucosuria and very pronounced polydipsia. In all the other cases the water intake was normal. Pathological study revealed that the tendency for fatty livers with degenerative changes

were most frequent with the type of obesity shown in Fig. 3 (38/56). Spontaneous deaths were, also, most common in this group.

During pregnancy and lactation in this strain of mice there was an enhanced preference for fat (Fig. 5).

## Discussion.

It should be pointed out that these experiments were not performed with "true" free selection of food but with free selection of three different diets.

In all the animals studied sofar — both control and obese mice — the desire for the high protein diet was absent or extremely low. The other two types of diets contained both 25 % protein, an amount apparently sufficient to satisfy the protein requirement. Consequently, no signs of protein deficiency were observed in these studies.

There was a marked variability among the obese mice with regard to the preference for carbohydrate and fat which, in turn, influenced the further course of the obesity. In most cases obesity could develop on the standard diet. In some of the injected animals, however, obesity did not develop on the standard diet but only on the free selection diet. For example, one mouse (Fig. 4) did not develop obesity after several months on the standard diet. There was only a slight increase in weight over its controls. When placed on free selection, the mouse gained almost 10 grams in weight. The controls remained unchanged. This higher weight level was not maintained when the animal was returned to the standard diet. Therefore, the weight dropped to the previous level. When returned once again to free selection the weight increased to the higher level.

Most frequently, however, the injected animals developed obesity on the standard diet. Free selection feeding in these cases caused a weight gain varying considerably from animal to animal (Fig. 2). While gaining additional weight, most of the obese mice showed a marked preference for the high fat diet. In many cases this diet was the only energy source. Therefore, the mice only consumed fat (75 %) and protein (25 %). When the new higher weight level was reached, many of the mice also began to eat the high carbohydrate diet (Fig. 3, 31/55). In this case it was impossible to judge if the decreased preference for fat was because a high weight level was reached. The failure of further

weight gain could have been secondary to diminished fat intake caused by some unknown circumstance. In other instances the obese mice continued with the high fat diet even after the higher weight plateau was reached (Fig. 3, 38/56). As seen in the figure this particular mouse sometimes reduced its caloric intake — and the percent fat intake — so much that weight loss occurred. After some days the caloric intake was again increased, and the higher weight level regained. No definite explanation for this variability is available. It is possible that the liver could be a limiting factor when high fat intake has lasted over a long period. Pathological examinations support this idea (BROBECK, TEPPERMAN and LONG 1943, DRACHMAN and TEPPERMAN 1954). Fatty livers have been frequently encountered in these animals. Other factors influencing the variability of the caloric intake probably exist.

All the mice studied in these experiments have been older than one year, and the injection of goldthioglucose was made at least 8 months prior to these studies. Therefore, on the standard diet, the weights of the animals had reached a steady state. As pointed out in a previous study (LARSSON and STRÖM 1957) the duration of the obesity plays a role with regard to the quantitative aspects of food intake. ANLIKER and MAYER (1956), continuously recording food intake over a long period of time in goldthioglucose mice, obtained a somewhat different eating pattern from that of LARSSON and STRÖM (1957). No doubt this depended both upon differences in the ages of the mice and the duration of obesity in the two studies. It seems possible then that mice in the earlier stages of obesity have other food preferences. Preliminary studies at this laboratory also point in that direction.

The experiments show the variability of both the quantity and the quality of the food intake in this type of obesity, in spite of the fact that the obesity has the same etiology, namely the injection of goldthioglucose. As shown by MARSHALL, BARRNETT and MAYER (1955) and confirmed by LARSSON and STRÖM (1957) this substance produces a specific lesion in the ventromedial parts of the hypothalamus. This area is considered to be the satiety part of the "feeding center" (ANAND and BROBECK 1951 a and b). According to MAYER (1952) the "feeding center" contains glucoreceptors. Other evidence for the existence of glucoreceptors has been given by DUNÉR (1953) and FORSSBERG and LARSSON (1954 and 1955). As suggested by MARSHALL et al. (1955) the glucoreceptors are damaged by the goldthioglucose. The variability in the qualita-

tive choice of food in the obese animals, then, could be due to varying extent of the lesions in the satiety part of the "feeding center", presumably of the glucoreceptors. SOULAIRAC (1956) has given some evidence that the brain contains separate regulatory mechanisms for both the quantity and the quality of the food intake. It is not known at present if these two parts are able to work independently of each other, as presumably is the case for the satiety and appetite parts of the "feeding center" (BROBECK, LARSSON and REYES 1956). Probably the former regulatory mechanisms are more closely related to each other, since, as mentioned above, an animal must change over to a diet containing more fat in order to gain weight.

This was also demonstrated in normal female mice of the same strain, during the pregestation, gestation and lactation periods. In the course of pregnancy the mice on free selection had an increased preference for fat. After the cessation of lactation the animals showed less interest in the high fat diet. The results of RICHTER and BARELARE (1938), studying essentially the same thing in rats, are, therefore, confirmed by the present studies.

The observations on females show a striking similarity to the free selection studies in the obese mice. Therefore, in normal mice under increased demands for energy, an adequate caloric intake for maintaining body weight, is made possible by a high intake of fat. In the obese mice, then, a higher weight level could only be reached by the same type of food preference. The simplest explanation for the increased desire for high fat diets is, of course, that fat has a higher caloric density than carbohydrate and protein. STROMINGER, BROBECK and CORT (1953), working with "hypothalamic" obesity in rats found that the obese animals did not adapt their food intake in the manner of normal rats when fat or cellulose were added to the diets. In normal rats, however, an adaptation took place. In this case the specific dynamic action of the food was suggested to be an important intrinsic food factor (STROMINGER, and BROBECK 1953). The specific dynamic action of the food is, of course, in many ways related to the energy content of the food.

The experiments of LUNDBAEK and STEVENSON (1947) and LARSSON and STRÖM (1957) and those reported here seem to indicate that the glucostatic theory introduced by MAYER (1952) and the theory suggested by STROMINGER and BROBECK (1953), both giving explanations for the normal regulation of food intake,

are likely to be of equal importance. The glucostatic regulation probably is working on a short time basis, while the specific dynamic action of the food influences food intake over longer periods.

The variability in the preference for different diets in the obese mice, however, indicates that other factors are capable of interfering with the central mechanisms regulating food intake. As an example the palatibility of the food plays an important role (KENNEDY 1953) together with the above mentioned influence of age and stage of the obesity. Further, it has been shown by FENTON and DOWLING (1953) that different strains of mice react differently with regard to body weight when placed on a high fat diet. MAYER and JONES (1953) have studied the hereditary hyperglycemic obese mice and found that these animals only develop obesity on a diet rich in carbohydrate.

The normal mice used in these studies did not increase their caloric intake when given free choice of food. In general, carbohydrate and fat contributed equally to the diet in these animals together with the obligatory 25 % protein (Fig. 1).

The differences between obesity with varying etiology have recently been reviewed by LARSSON (1956). In the present study the obesity was induced only by goldthioglucose, and the results showed marked variations in food preferences and further course of the obesity. It is, therefore, impossible to outline the general characteristics for the qualitative aspects of food intake. In man the treatment of obesity by dietary means has been applied with varying success. Different food compositions have been suggested from time to time. It is, however, important to bear in mind that "Lack of success in the treatment of obesity requires that we maintain a flexible attitude towards these patients" (GORDON 1954).

The water intake in goldthioglucose obesity has previously been studied in animals kept on standard diet (LARSSON and STRÖM 1957). No changes from the normal regulation of water intake were observed, and the present experiments confirm these results. There were some exceptions from these in the obese animals that developed clinical signs of diabetes with marked glucosuria. In these cases the water intake was extremely high but regarded as secondary polydipsia.

## Summary.

1. The preference for different diets has been studied in adult mice, both normal and those made obese by a single injection of goldthioglucose.

2. The normal mice did not show any changes in weight when transferred from standard to free selection diets. On free selection the caloric intake of fat and carbohydrate was about equal.

3. The obese mice showed marked variations in weight gain and food preferences. While gaining weight no carbohydrate was eaten in most cases.

4. In some cases obesity did not develop on the standard diet but only on the free selection diet.

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